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INJURY AND THE IMPROVED RECOVERY OF COLIFORM BACTERIA IN DRINKING WATER

by

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16. ABSTRACT

A study was conducted to answer basic questions about the fate and handling of stressed indicator bacteria in aquatic environments. The importance of stressed indicator bacteria in assessing water quality has been gaining attention as microbiologists have discovered the inaccuracy of present tools for recovering indicator bacteria in water. The study examines the effects of laboratory manipulations on recovery of injured indicator bacteria. These manipulations include exposure to diluents, choice of enumeration medium, and membrane filter surface pore morphology.

Results indicated that diluent composition was of little importance, provided the diluent was cold (4°C) and exposure was brief (<30 min.), and that the most commonly used media recovered less than 30% of an injured cell population. Based on these results, a new membrane filter medium, m-T-7, was developed that recovered 86% to 99% more laboratory-injured coliforms and 3 times more coliforms from drinking water than did m-Endo agar. The m-T7 agar also recovered 43% more verified coliforms from 67 surface and drinking water samples than did the standard m-Endo membrane filter technique. Recoveries of fecal coliforms on m-T7 medium were 3.1 times greater than the standard m-FC method and 1.7 times greater than the two-layer enrichment, temperature acclimation procedure.

Comparisons of verification techniques for the membrane filter total coliform method suggest that standard procedures may result in significant underestimations of indicator densities, particularly in waters where coliforms are stressed or injured.

Laboratory and field data indicated that copper at levels normally found in drinking water could injure coliform bacteria. Results showed that 90% injury could occur at a copper concentration of 0.050 mg/l.

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FOREWORD

The U.S. Environmental Protection Agency was created because of increasing public and government concern about the dangers of pollution to the health and welfare of the American people. Noxious air, foul water, and spoiled land are tragic testimonies to the deterioration of our natural environment. The complexity of that environment and the interplay of its components require a concentrated and integrated attack on the problem.

Research and development is that necessary first step in problem solution, and it involves defining the problem, measuring its impact, and searching for solutions. The Municipal Environmental Research Laboratory develops new and improved technology and systems to prevent, treat, and manage wastewater and solid and hazardous waste pollutant discharges from municipal and community sources, to preserve and treat public drinking water supplies, and to minimize the adverse economic, social, health, and aesthetic effects of pollution. This publication is one of the products of that research and is a most vital communications link between the researcher and the user community.

Stress to indicator bacteria has long been recognized as a problem in the accurate assessment of potable water supplies. This investigation was initiated to further study the causes of injury, factors that affect injured organisms, and to develop new methods to enumerate injured coliforms. This report will be of benefit to treatment plant operators, health authorities, and administrators in making decisions concerning the treatment and quality of this nation's potable waters through an expanded understanding of injured microorganisms and the impact they have on water quality assessments.

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ABSTRACT

A study was conducted to examine the effects of the following laboratory variables on the enumeration of injured and undamaged coliform bacteria in drinking water: (a) diluent composition, temperature, and time of exposure, (b) media of various formulations used in enumerating Gram-negative bacteria, and (c) surface pore morphology of membrane filters.

As a result, a new membrane filter medium, m-T7 agar, was developed to improve the recovery of injured total coliforms from drinking water. The m-T7 agar also recovered an average of 43% more verified coliforms from 67 surface and drinking water samples than did the standard m-Endo membrane filter technique. From drinking water, m-T7 agar recovered nearly 3 times more coliforms than m-Endo agar. This agar was also evaluated as a fecal coliform medium and was found to produce consistently higher fecal coliform counts than any of the other methods tested when the agar was preincubated for 8 h at 35° to 37°C.

Verification techniques were compared for the membrane filter total coliform method using the standard lauryl tryptose broth (LTB) procedure and the B-galactosidase and cytochrome oxidase (B-gal/CO) method. Of the 682 presumptive coliforms tested from all types of water samples, 577 (84.6%) were verified using the B-gal/CO method, whereas only 402 (58.7%) produced gas in LTB.

Studies were also initiated to investigate the interactions between standard plate count (SPC) bacteria and coliform organisms, and to develop a preservation technique to eliminate these interactions during a 5-day transport/storage interval. In spiked samples, SPC bacteria reduced coliform densities by more than 3 logs within 8 days. A solution of sodium benzoate (10 g/l), yeast extract (1 gm/l), and peptone (1 g/l) effectively preserved Escherichiia coli densities (80% to 100% viability) over a 5-day period. Low levels of copper in distribution water were shown to be responsible for coliform injury when chlorine was absent. A statistical model was developed to predict coliform injury on the basis of copper, temperature, pH and alkalinity. The relevance of these findings are discussed in relation to sample collection and transport procedures.

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PREFACE

Over the past three years we have conducted investigations relating to injury of indicator bacteria. The results reported here deal primarily with injury to total coliform bacteria in drinking water. Some of the areas investigated include: effect of surface pore morphology on injured total coliform recovery, effect of media composition on enumeration of injured indicator bacteria, development of a medium specifically designed to enumerate injured coliforms in drinking water, comparison of verification techniques for total coliform bacteria, and preliminary investigations into the injurous interactions between heterotrophic plate count bacteria and coliforms which occur during transport and storage and methods for eliminating these interactions. In addition, our studies led us into areas not directly related to drinking water. We found that diluent composition and temperature was important when enumerating injured cells. While diluents are not routinely used in the assessment of potable waters, on occasion a contaminated sample may need to be diluted. Likewise, evaluation of m-T7 agar as a fecal coliform medium was a natural extension of the work done on the total coliform procedure since fecal coliform analyses may occasionally be performed on contaminated distribution samples.

The first six chapters of the study give in depth reports covering various aspects of our investigations. These are presented in order to give detailed descriptions of experimental design and results. Chapter seven is a review, and is intended to give an overview of injury in drinking water. This chapter will be of interest to administrators and policy makers. Our conclusions and recommendations are included at the end of this chapter.

The appendix at the end of the report contains the evaluations of five laboratories using m-T7 agar. While these evaluations are generally positive, there is a great need to test this medium on more actual drinking water samples from different localities. We expect that through the use of this medium, a better understanding of injured coliform bacteria in drinking water will be possible.

SECTION 1

INFLUENCE OF DILUENTS, MEDIA, AND MEMBRANE FILTERS ON THE DETECTION OF INJURED, WATERBORNE COLIFORM BACTERIA

SUMMARY

Pure cultures of *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter aerogenes*, and *Citrobacter freundii* were injured (> 90%) in water from a dead-end section of the Bozeman, Montana, distribution system. The effects of the following laboratory variables on the enumeration efficiency of injured and undamaged control cells were examined: (a) diluent composition, temperature and time of exposure, (b) media, using various formulations employed in enumerating Gram negative bacteria and (c) surface pore morphology of membrane filters. The addition of peptone or milk solids to diluents and a low temperature (4°C) maximized the recovery of injured cells, but it had little effect on undamaged cells. Control cells were recovered with high efficiencies on most media tested, but recoveries of injured cells ranged from 0% to near 100%. Most of the media commonly used in water analysis recovered less than 30% of injured cells. This result was partly explained in by the sensitivity of injured bacteria to desoxylcholate concentrations greater than 0.01% while control cells were unaffected by 0.1%. Membrane filter surface pore morphology (at 35°C) had negligible effect on total coliform recoveries. Recommendations are made regarding procedures to improve the recovery of injured coliforms by routine laboratory practices.

INTRODUCTION

The detection of coliform bacteria in drinking water and wastewater can be inhibited by a variety of factors, including excessive numbers of heterotrophic bacteria, turbidity, and a process known as sublethal injury (40,94). As a result, the actual number of indicator organisms present may be underestimated. Sublethal injury has been established as an important factor in determining the safety of foods exposed to physical and chemical treatments that damage indicator bacteria (15,53). Such debilitated organisms are often unable to grow on selective media (72), but they can regain that capability through a resuscitation process (7,8,15,17,53) under non-restrictive conditions. The concept of sublethal injury was first associated with the suppression of waterborne indicator bacteria when it was noted that coliform enumeration data from waters containing toxic wastes or chlorine were consistently higher by the multiple tube fermentation most probable number (MPN) method than by the membrane filtration (MF) procedure (12,17,53,107). More than 90% of indicator bacteria present may become injured when exposed to natural waters for less than 1 week (7,8). Injury is an important factor in underestimating numbers of waterborne indicator bacteria and may lead to inaccurate public health assessments.

Injury in the aquatic environment may be related to a number of factors, including time and temperature of exposure, disinfection levels, strain of organism, concentration of nutrients, presence of heavy metal ions, antagonistic standard plate count bacteria and possibly other undefined chemical and physical parameters (7,15,40,86,119,120,123). In addition, laboratory manipulations involving exposure to diluents, selective media, and membrane filtration may cause further underestimations of bacterial densities in aquatic environments. The importance of diluent composition in the accurate bacteriological analysis of water samples was pointed out in earlier studies (16,119). More recent reports (7,12,56,59,94,109,118,120) have supported the previous conclusions and suggested the value of low concentrations of organic additives such as peptone, tryptone, or milk solids. In most studies, however, little attention has been given to injured bacteria. Selective media have likewise been reported as a factor that causes variable recovery efficiencies of indicator bacteria (8,31,32,43,49,94,103). Bacteria injured in water have been shown to be more sensitive to selective ingredients in various enumeration media (8,31) because of damage to the bacterial envelope (123). This realization stimulated the successful development of resuscitation procedures and media for the improved enumeration of injured fecal coliforms, particularly in chlorinated wastewater effluents (7,8,44,68,110). A new section describing these methods entitled, "Stressed Organism Recovery" (Section #921) has been added to the 15th edition of Standard Methods for the Examination of Water and Wastewater (2). Recently, the choice of membrane filter type has also been reported to affect the recovery of indicator organisms (39,108,112). Sladek, et al. (108) reported that optimal recovery of fecal coliform bacteria on membrane filters with the high-temperature test (2) was a function of the surface pore morphology of the filters, but this concept was not applied to injured total coliforms grown at 35°C. However, coliform bacteria exposed to chlorine levels found in drinking water for 10 minutes or more may be recovered less efficiently by membrane filtration than on spread plates of the same medium (48).

There has been general agreement that characteristics of diluents, selective media, and membrane filters influence the recovery efficiency of waterborne indicator bacteria, but little attention has been devoted to understanding the involvement of prior cellular injury in these laboratory manipulations. The research reported here examined the recovery of coliforms both before and after substantial cellular injury in drinking water with respect to: (i) The composition, exposure time and temperature of diluents, (ii) the composition of various media commonly used in water quality testing, and (iii) the surface pore morphology of membrane filters at 35°C.

MATERIALS AND METHODS

Cultures

Cultures were obtained from surface and distribution water by the membrane filter technique (2). The isolates were identified using the API 20E system (Analytab Products, Plainview, N.Y.) as *Escherichia coli* (2 strains), *Klebsiella pneumoniae*, *Enterobacter aerogenes* and *Citrobacter freundii*. The cultures were streaked periodically on m-Endo agar (Difco), and a colony with metallic sheen was selected. Cultures were stored on slants of tryptic soy broth (Difco) plus 0.3% yeast extract (Difco), 1.0% lactose (Difco) and 1.5% agar (Difco) (TLY agar) at 4°C.

Organism preparation and laboratory injury

Eighteen hour stationary phase organisms grown in TLY broth (tryptic soy broth without glucose, Difco, supplemented with 1% lactose and 0.3% yeast extract) were harvested by centrifugation at 3000xg in a Sorvall Model RC2-B centrifuge, washed twice in sterile refrigerated reagent-grade water (obtained with a Millipore, Milli-Q reagent-grade water system supplied with single-distilled water) and resuspended to concentrations of approximately 10^9 cfu/ml. Following washing, at the beginning of each experiment, cultures were plated out to assess if injury had occurred due to preparatory procedures. Because of the short washing time and use of cold reagent-grade water no injury occurred in any of the preparatory procedures. Injured populations of coliforms were obtained by placing washed cells into a membrane dialysis chamber (75) immersed in water drawn from a dead-end main of the Bozeman, Montana, Drinking water distribution system. Bacterial populations became 90% injured within 2-4 days. Various water characteristics were monitored including conductivity, pH and free residual chlorine. None of these characteristics correlated uniquely to the rate or occurrence of injury in coliforms indicating the complex nature of aquatic stress. Samples were periodically withdrawn and tested for injury by comparing viable counts obtained by the spread plate technique (2) on TLY agar (TLY broth + 1.5% agar) and TLY agar with 0.1% desoxycholate (TLY-D). The percent injury was calculated by the following equation:

$$\%\ \text{injury} = \frac{\text{TLY count} - \text{TLY-D count}}{\text{TLY count}} \times 100$$

A population was considered "injured" when at least 90% of the cells failed to grow on the selective medium (TLY-D).

Effect of diluent composition on coliform recovery

Five diluents were tested with injured and non-injured *E. coli* suspensions at 4° and 23-24°C (room temperature) to determine the effect of diluent composition, temperature and time of exposure on the recovery of injured coliforms from water. Diluents used were: reagent-grade water, phosphate buffer (2), 0.1% peptone water (2), phosphate buffer amended with 0.1% peptone, and 1.0% milk (Difco). Diluents were sampled in triplicate at 30 minute intervals by the membrane filter technique (2) using Millipore HC filters. Filters were placed on m-Endo agar (Difco) and incubated 22-24 hr at 35°C. The percent recovery was determined by dividing the average count obtained throughout the experiment by the zero time count.

Effect of media composition on coliform recovery

Injured and non-injured coliforms were plated on various media to determine which formulation permitted the best recovery from water. The media tested included: triple sugar iron agar, tergitol 7 broth without indicator, lactose broth, E.E. broth, brilliant green broth, Levine's eosin methylene blue, lauryl tryptose broth, m-Endo broth, desoxycholate lactose agar, brilliant green bile 2% broth, violet red bile, m-FC broth, eosin methylene blue agar, MacConkey agar, GN broth and XLD agar (all from Difco). Also tested were nutrient alginate (33), boric acid broth (24), purple serum agar (86), minerals modified glutamate (86), TLY, TLY-D, TLY plus 0.1% tween 80 (Difco) and 3V agar (Millipore Corp.). To all broths 1.5% agar (Difco) was added to make a solid medium. Plates were prepared at least 4 h prior to inoculation to allow for adequate drying. Appropriate dilutions of the organisms were made in sterile reagent-grade water at 4°C. Quintuplicate plates of each medium were inoculated by the spread plate technique (2) and incubated for 18-24 h at 35°C except boric acid agar which was held at 35°C for 48 h. Following incubation the average count from each medium was compared to the average count obtained on TLY to determine the percent recovery.

Effect of membrane filter type on the recovery of coliforms

Experiments were conducted to compare recovery of injured and non-injured *E. coli* on Millipore HC and HA membrane filters. Appropriate dilutions of the organism were made in sterile reagent-grade water at 4°C and processed within 30 min. Five, 1 ml samples were filtered through the membrane filters and placed on TLY and TLY-D. Dilutions were also plated on TLY and TLY-D by the spread plate technique and all the plates were incubated at 35°C for 18 h.

Throughout the course of this study a quality assurance program was followed as outlined in *Standard Methods for the Examination of Water and Wastewater* (2) and *Microbiological Methods for Monitoring the Environment* (10).

RESULTS

Effects of diluents on the recovery of injured coliforms

The enumeration efficiency of injured and healthy suspensions of E. coli was examined following exposure to five diluents. Sample dilutions are generally required in the analysis of polluted waters and phosphate dilution water or 0.1 percent peptone water are the recommended diluents (2). What effect these diluents and others might have on recovery of injured and healthy suspensions of E. coli was investigated in relation to time and temperature of diluent exposure. Injured organisms diluted in various diluents at 24°C varied widely in the efficiency of recovery during the 90 minute span of the experiments (Fig. 1). Injured bacteria held for 90 min in phosphate buffer or reagent-grade water were recovered at only 30-35% of the initial levels. Dilutions made in peptone water or phosphate buffer with 0.1% peptone were the most stable, yielding recoveries of 80-90% after 90 min of exposure. Repair of the injured coliforms occurred in milk at 24°C increasing the bacterial counts on m-Endo agar greater than 20%. Densities of uninjured control cells remained virtually unchanged throughout the experiment regardless of the diluent used.

When the diluents were used at 4°C the effect of the different formulations (Fig. 2) was much less than at 24°C. The recovery response of the injured cells was very similar to the response of uninjured cells when exposed in each diluent. This was particularly true in the first 30 minutes.

Effect of various media on recovery

The recovery efficiency of injured and control coliforms was tested on a variety of selective media. Media were chosen from among those used in various applications where enteric bacteria are enumerated. The results presented in Table 1 indicate that uninjured control cells were recovered at nearly 100% efficiency on most of the media tested, while injured cells were recovered at lower rates. The media are arranged in three groups based on the response of the injured cells.

Some of the media most commonly used in water testing were evaluated on a year-round basis. The ranges and average recovery rates for these media were calculated from 7 repetitions using 5 coliform species. Four genera of coliforms; E. coli, (2 strains) K. pneumoniae, C. freundii and E. aerogenes, were used to determine if variations occurred in recoveries of different coliforms. No substantial variation occurred between the different genera in their response to different media compositions. Eleven of the sixteen most suppressive media (groups II and III, Table 1) contain desoxycholate or bile salts. Because of that observation, an experiment was done to examine the relationship between desoxycholate concentration and recovery efficiency on a complete medium. The results in Figure 3 show that while uninjured cells are virtually unaffected by desoxycholate concentrations of less than 0.1%, injured cells are severely inhibited at all concentrations greater than 0.01%.

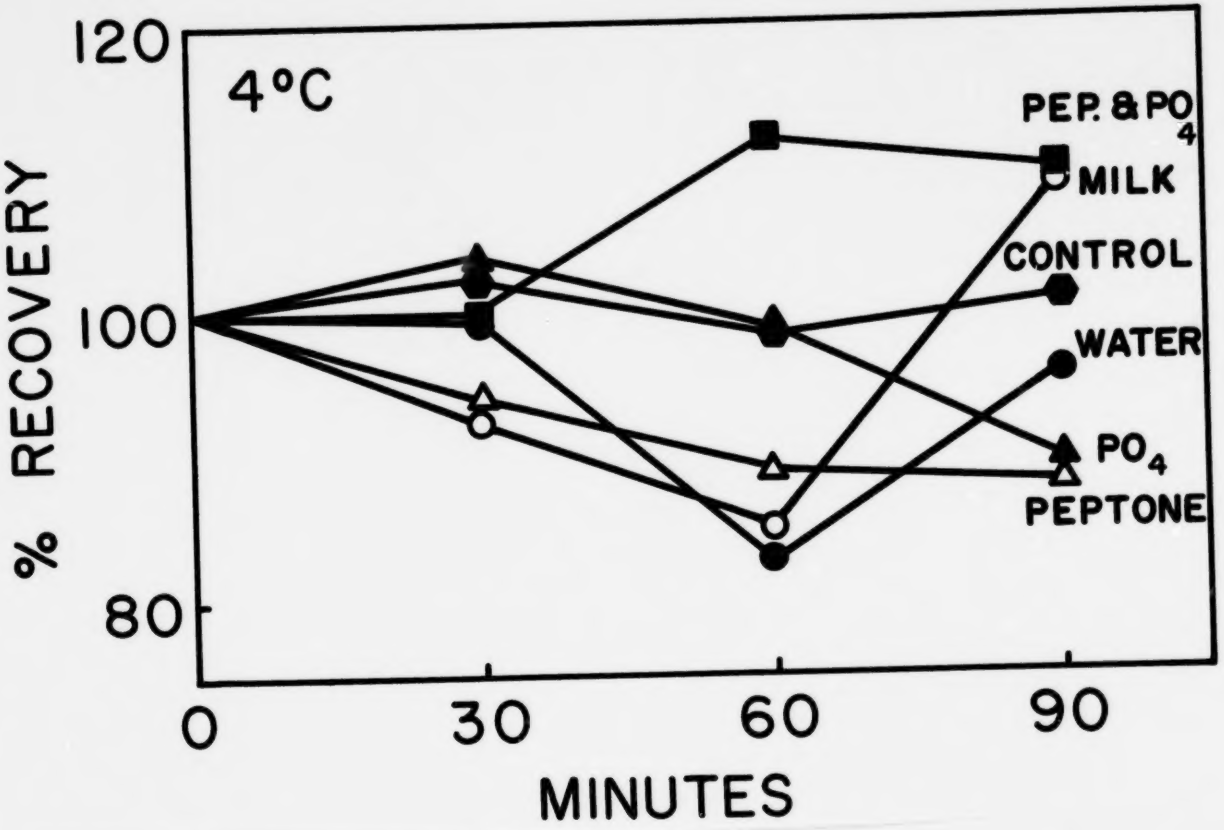

Figure 1. Effect of diluent composition and exposure time on the recovery of injured (90%) and uninjured control E. coli suspensions at 4°C. The control line is an average of all data obtained when uninjured cells were exposed in each diluent. Diluents are 1.0% milk, 0.1% peptone, reagent-grade water, phosphate buffer and phosphate buffer amended with 0.1% peptone. Values are a mean of three experiment replications.

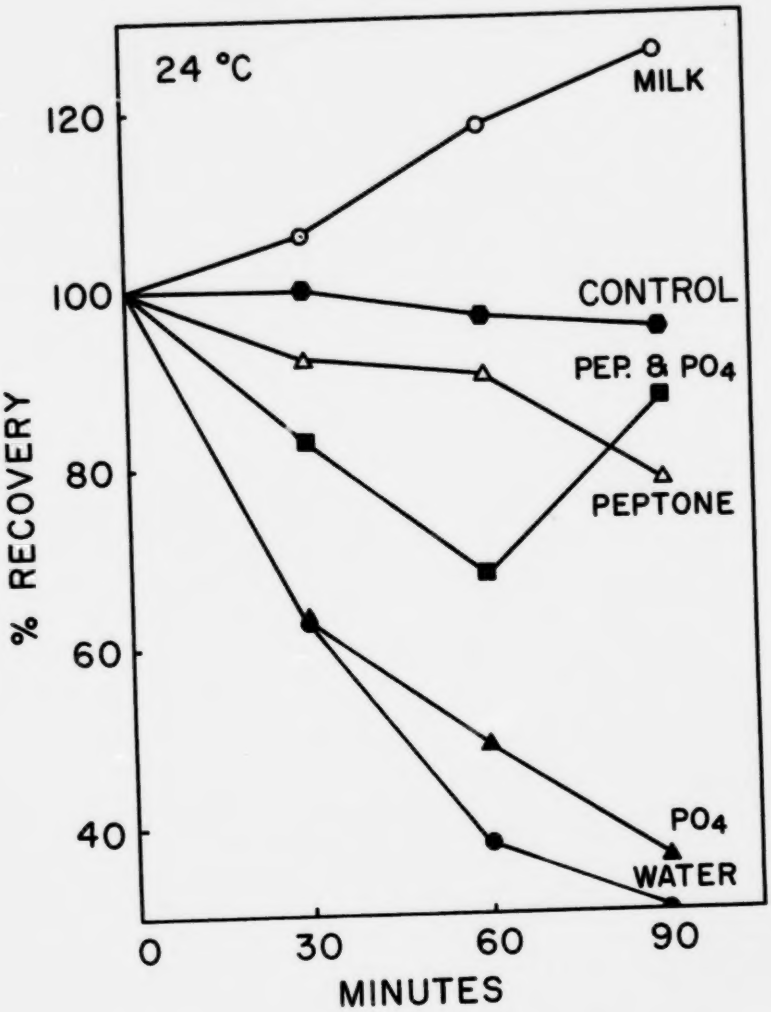

Figure 2. Effect of diluent composition and exposure time on the recovery of injured (90%) and uninjured control *E. coli* suspensions at 24°C. The control line is an average of all data obtained when uninjured cells were exposed in each diluent. Diluents are 1.0% milk, 0.1% peptone, reagent-grade water, phosphate buffer amended with 0.1% peptone. Values are a mean of three experiment replications.

Table 1. MEDIA AND THE RECOVERY OF INJURED AND HEALTHY COLIFORMS[a] FROM WATER

Media	% Recovery[b] Injured[c]	Range[d]	Healthy	% Desoxycholate or Related Compounds[efg]
I				
Triple Sugar Iron	181		106	0
Nutrient Alginate	125		88	0
Minerals Modified Glutamate	99		106	0
Tergitol 7	86	71-101	99	0
Boric Acid	84		92	0
TLY + 0.1% Tween 80	72		--	0
II				
Lactose Broth	72	47-98	102	0
m-ENDO	66	30-102	93	0.1; 0.005[g]
Lauryl Tryptose	56	34-79	98	0.01[g]
Levines EMB	42	37-47	119	0
3V	39		95	N.A.[h]
Purple Serum	38		56	0
E E	38		106	2.0[e]
Brilliant Green Bile 2%	34	18-51	106	2.0[e]
Desoxycholate Lactose	26		94	0.05
III				
Eosin Methylene Blue	24	7-42	102	--
Violet Red Bile	12		99	1.5[f]
m-FC at 44.5°C	7	4-10	105	1.5[f]
MacConkeys	5		97	0.1[f]
GN	4		71	0.05
TLY-Desoxycholate	2		82	0.10
XLD	0		40	0.25

---

[a] Coliforms tested include: *Escherichia coli* (2 strains), *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter aerogenes*.

[b] $\% \text{ Recovery} = \frac{\text{cfu selective medium}}{\text{Cfu TLY}} \times 100$

[c] Injury was between 90 and 99%

[d] The range is calculated from 7 repetitions using 5 coliforms over a 1 year period

[e] Oxgall

[f] Bile Salts

[g] Lauryl sulfate

[h] not available

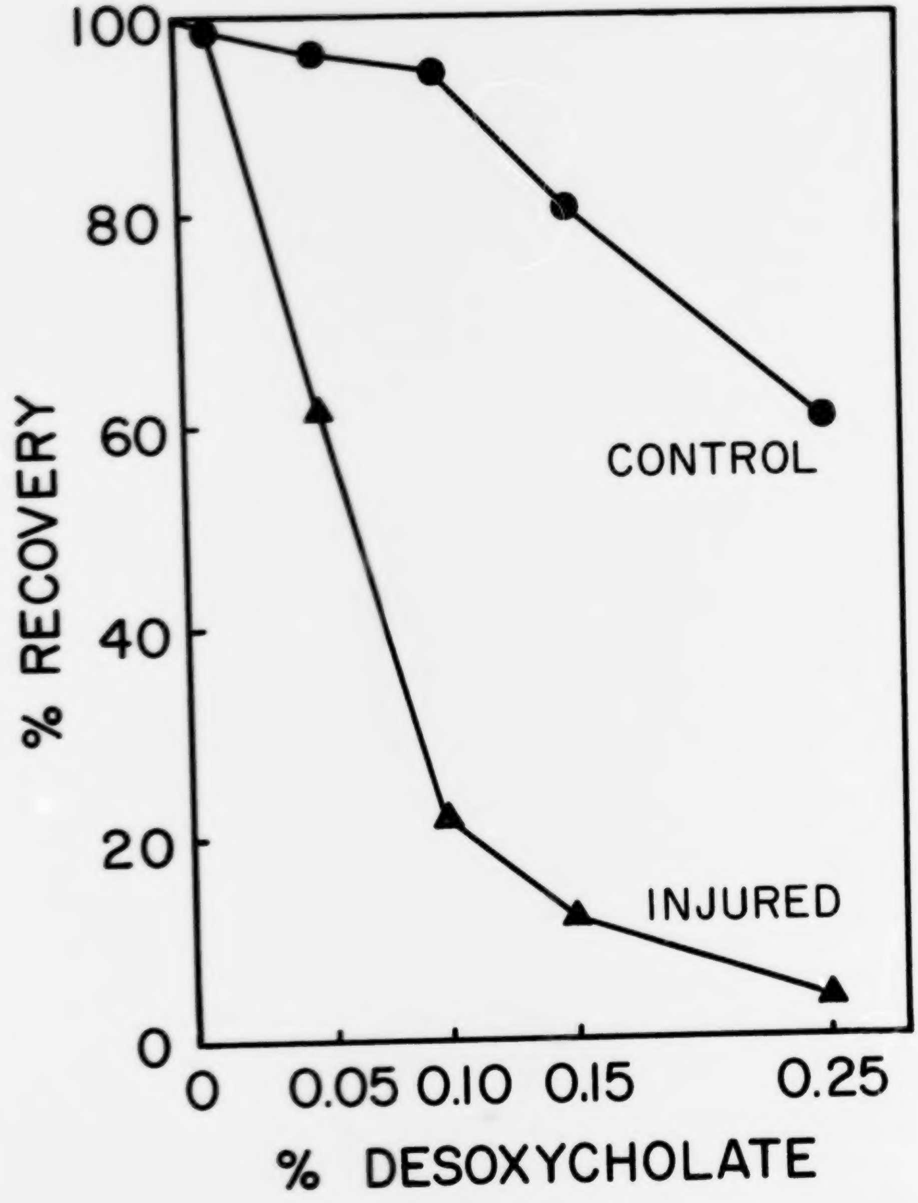

Figure 3. Effect of various concentrations of desoxycholate on the recovery efficiency of injured and control E. coli suspensions. Desoxycholate was added to TLY agar and the spread-plate method was used.

Membrane filter surface pore morphology and recovery

Populations of injured *E. coli* were enumerated on complete selective (TLYD) and complete non-selective (TLY) media using two filters. The filters were selected to examine the effect of the mean surface pore diameter on the recovery efficiency of injured *E. coli* at 35°C. The results (Table 2) indicate that the membranes with a 2.4 um surface pore opening (HC) and those with openings less than 1.0 um (HA) yielded nearly identical results. Also the orientation of the filters with respect to the grid markings made no difference. Similar results were seen when uninjured control cells were examined under the same conditions.

DISCUSSION

The dilution step, as demonstrated by the data in Figures 1 and 2, can greatly influence the enumeration efficiency of injured coliforms. Early studies done in 1927 (119) and 1932 (16) suggest a critical role of diluents in some bacteriological examinations. More recent reports have further emphasized this conclusion with non-stressed bacterial suspensions (56,59,94) and cells that were injured by freezing (118) and exposure to water (7,109,120). Diluents containing 0.1 or 0.05% peptone have been useful in recovery of attenuated organisms from food, industrial wastes and waters containing heavy metals (109), but bacterial multiplication can occur if the time between sample dilution and plating exceeds 40 min (38). Phosphate buffer containing magnesium phosphate (2) has been used to dilute samples containing metabolically injured cells. Samples held for more than 30 min have been reported to result in loss of viable cells (38). Because of these considerations *Standard Methods* (2) imposes a 30 min time limit on processing diluted samples at room temperature. Our findings point to the importance of the diluent composition, temperture and the time of exposure. If diluents are maintained at approximately refrigerator temperatures (c.a. 4°C) their composition and exposure time are likely to be of minimal impact on enumeration efficiency. However, substantially lower enumeration recoveries are associated with room temperatures and extended exposure times. The enrichment of diluents with low concentration of organic materials such as peptone, gelatin, tryptone, or milk has been demonstrated to be of value in the enumeration of aquatic bacteria (7,56,59,94,109,118-120). The results reported here indicate that these factors are even more important in situations where injured coliforms are enumerated. This consideration is of particular significance in applications such as the enumeration of coliforms in chlorinated drinking water and wastewater effluents where the disinfectant is known to cause substantial injury (12,17,72,103). Additional factors such as metals probably also act as stressors to waterborne indicator bacteria.

The selection of media to be used in determining coliform numbers in water and wastewater can also significantly affect results. Several recent studies (31,32,49,72) have shown that

Table 2. Effect of membrane filter surface pore morphology on recovery of coliforms.

	Healthy Cells[a]			Injured Cells[b]		
Enumeration Technique	TLY	TLY-D	% Injury	TLY	TLY-D	% Injury
Spread plate	62	49	21	321	8.4	97.4
Membrane filter HA[c]	68	44	35	309	11.8	96.2
Membrane filter HC[c]	92	60	35	327	13.0	96.0

[a] Counts are an average of two experiments of 5 replicates each (x 10^7 cfu/ml)

[b] Counts are an average of three experiments of 5 replicates each (x 10^6 cfu/ml)

[c] Millipore

various selective media yield markedly different coliform enumeration results. Substantially reduced plating efficiencies on selective media have been associated with coliforms damaged from exposure to injurious chemicals in the environment (7,8,12,103). Therefore, it is not surprising that coliforms injured in aquatic environments have been recovered with efficiencies of 10% or less on commonly used media (7,8,12,43,71). Our results (Table 1) demonstrate that in comparison to uninjured cells, injured coliforms yielded reduced plating efficiencies on a variety of selective media used for the recovery of Gram negative enteric bacteria. The ranking of the various media, shown in Table 1, agrees with results reported by others (8,12,,43,49) where fewer media were compared. Of practical significance, is the finding that media widely used for water quality estimations (m-Endo, m-FC, EMB, and MacConkey's) generally ranked in the lower half of all media tested with recoveries ranging from 30 to 70%. Lauryl tryptose broth had low recoveries of injured coliforms (avg. 56%, range 34-79), possibly accounting for reports of failing to find injured coliforms in water by the recommended resuscitation technique (35).

Little variation occurred between different genera of injured coliforms tested (E. coli, 2 strains; K. pneumoniae, C. freundii and E. aerogenes) in their response to different media compositions. However, seasonal variations in the physical and chemical properties of the distribution water used to injure the coliform organisms may be responsible for the large variations in recoveries of certain media (m-Endo 30-102%). Insight into these seasonal variations are not possible with the data collected in this report (see section VI).

In an attempt to explain the relatively poor performance of the media tested, the selective agents in the different formulations were noted. Seven of the eight media in group three (Table 1) having recovery efficiencies between 0 and 24% as well as four of the media in group two, contained either bile salts or desoxycholate. For that reason, we tested the recovery of both injured and healthy E. coli in various concentrations of desoxycholate added to a complete medium (Fig. 3). Control cells were virtually unaffected by concentrations of 0.1% or less while injured cells were severely inhibited by greater than 0.05% desoxycholate. Not surprisingly, concentrations of bile salts or desoxycholate in media that were most inhibitory to injured coliforms (group III, Table 1) were between 0.05 and 2.5%.

A recent report (123) concludes that a major location of the injury in water-injured coliforms is in the cell envelope. Such cells become more sensitive to surfactants, including bile salts and desoxycholate (123). The formulation of an improved medium, based on an understanding of the physiology of injury, is now possible. This medium must however, be highly selective for use in the analysis of water and still recover the injured coliforms efficiently. The feasibililty of this suggestion is supported by previous work (8), indicating that injured fecal coliform bacteria can recover the ability to grow on selective media (44,68,99,110).

Dissatisfaction with the performance of membrane filters was summarized by reports (16,39) that some types of membrane filters were more effective in the recovery of waterborne fecal coliform bacteria from a variety of sources. A subsequent study by Sladek, et al. (108) identified surface pore morphology as the critical physical characteristic of membrane filters that influenced fecal coliform recovery on selective media in the high temperature (44.5°C) test. This conclusion was not, however, extended to the enumeration of total coliforms at 35°C or to injured waterborne indicator bacteria, although it has been reported that chlorine-injured coliforms may be recovered less efficiently with membrane filters than by spread-plating (48). For that reason, experiments were carried out to compare the recovery of highly injured _E. coli_ suspension on membranes of large (2.5 µm) and small (less than 1.0 um) surface pore openings with spread plates using the same media. The results (Table 2) showed that membrane filter surface pore morphology is not important in the recovery of injured total coliform bacteria since the counts with both filters and spread plates were comparable. This conclusion is consistent with the results of other reports (69,113) where the same filters were used, but the level of injury was not controlled or reported.

Reversible bacterial injury caused by stresses in aquatic environments (7,8,12,71) is an important factor contributing to the suppression of indicator bacterial enumeration (17,44,68,99,110,120). The results presented in this report have demonstrated that diluent composition, exposure time, and temperature reduce the recovery efficiency of injured coliform bacteria. In addition, media can play a critical role since many of the currently accepted media formulations used are detrimental to recovery of injured coliforms from water. These findings represent a supportive rationale for the development of new methodologies allowing a more complete enumeration of injured coliforms in potable water and providing more accurate and dependable water quality information.

SECTION 2

A NEW MEDIUM FOR THE IMPROVED RECOVERY OF COLIFORM BACTERIA FROM DRINKING WATER

SUMMARY

A new membrane filter medium was developed for the improved recovery of injured coliforms from drinking water, the new medium termed m-T7, consists of 5.0 g proteose peptone #3, 20 g lactose, 3.0 g yeast extract, 0.4 ml tergitol 7 (25% solution), 5.0 g polyoxyethylene ether W1, 0.1 g brom thymol blue, 0.1 g brom cresol purple and 15 g agar per liter of distilled water. Additional selectivity may be obtained by aseptically adding 0.1 ug/ml penicillin G to the medium after autoclaving. In laboratory studies m-T7 agar recovered 86-99% more laboratory-injured coliforms than m-Endo agar. m-T7 agar also recovered an average of 43% more verified coliforms from 67 surface and drinking water samples than the standard m-Endo membrane filter technique. From drinking water, m-T7 agar recovered nearly 3 times more coliforms than m-Endo agar. Less than 0.5% of the colonies on m-T7 agar gave false-negative reactions while over 70% of the typical yellow colonies from m-T7 agar produced gas in lauryl tryptose broth. Most of the verified coliforms isolated on m-T7 agar belonged to one of the four common coliform genera: Escherichia, 17.6%; Klebsiella, 21.7%; Citrobacter, 17.3%; Enterobacter, 32.2%. The results demonstrate that m-T7 agar is superior to m-Endo agar, especially for the isolation of injured coliforms from drinking water.

INTRODUCTION

The 15th edition of Standard Methods for the Examination of Water and Wastewater (2) specifies two methods for the microbiological analysis of potable water, the most probable number (MPN) and the membrane filter (MF) method. The MF technique has gained wide acceptance because the procedure is simple, rapid, precise, and gives definitive results. However, factors such as turbidity (36,40,50,63), high numbers of noncoliform bacteria (20,21,40,42,54,62,116,117), and membrane filter type (78,108) may severely influence the sensitivity of the procedure. In addition, the media specified for use with the MF technique, m-Endo agar, or m-Endo LES agar, have several shortcomings including: (i) low recoveries of injured coliforms (29,75-78); (ii) poor detection and differentiation of coliforms from non-coliforms (29,35,102); and (iii) uncertainty about the availability of high quality basic fuchsin (Geldreich, personal communication). As a result, many investigators have proposed alternative MPN or MF methods (27,34,57,58). However, an MF medium has not been found to be clearly superior to m-Endo agar and capable of isolating injured coliforms from drinking water.

Recently, we reported that some laboratory procedures currently used in water analysis may further reduce the recovery of injured coliforms (78). Included in that report was the observation that the majority of selective media used to isolate Gram-negative bacteria recovered 30% or less of the injured coliforms. However, Tergitol 7 agar, recovered between 71 and 100% of the injured coliforms tested.

Tergitol 7 agar was first introduced by Chapman in 1947 (19). The medium produced a consistent and characteristic colonial morphology with Escherichia coli, Enterobacter aerogenes and other Gram negative bacteria. Tergitol 7 (sodium heptadecyl sulfate) also inhibited many Gram positive bacteria including Staphylococcus aureus, Bacillus subtilis and Bacillus cereus (89). This medium was later modified to include 0.04% 2,3,5-triphenyl tetrazolium chloride (TTC) and was recommended as the medium of choice for the quantitative detection of E. coli in rat feces and drinking water (60,101). In more recent studies, Tergitol 7 medium (without TTC) recovered slightly more E. coli from chlorinated waters than either Teepol 610 or sodium lauryl sulfate media (57).

In this report we describe a further modification of Tergitol 7 agar to improve its selective and differential properties. This modified medium, termed m-T7, was superior to m-Endo agar for recovering coliforms from drinking water.

MATERIALS AND METHODS

Study area and sample collection

Drinking water samples were collected from the distribution system of two small communities near Bozeman, MT. The first drinking water system serviced 2,000 residents and consisted of both surface and well water that was only intermittently chlorinated. The second system serviced 8,000 residents and consisted of a network of 8 unchlorinated wells. Raw water samples were collected from the East Gallatin River approximately one half mile (0.8 km) downstream from the outfall of a sewage treatment plant. Laboratory chlorinated samples were prepared in two ways: (i) surface water was treated with 1.0 mg/l free residual chlorine (avg. pH, 7.7; avg. temperature, 10°C, prepared daily from stock solutions of bleach; (ii) chlorinated distribution water (free residual chlorine varied from 0.5-1.2 mg/l chlorine, avg. pH 7.6, avg temperature 10°C) was mixed with 10% surface water. Both preparations were dechlorinated with sodium thiosulfate (0.008%) after 10 minutes contact (2).

Water samples were collected in sterile 2 l polypropylene containers with (drinking water) or without (surface water and samples for laboratory chlorination) added sodium thiosulfate (0.008%) (2). Samples were placed on ice and transported to the laboratory within 1 h and analyzed within 5 h after collection.

Media development

A number of selective and differential compounds were tested to determine which agents allowed the recovery of stressed coliforms. Coliforms were injured by placing washed cultures of *E. coli*, *Klebsiella pneumoniae*, *Enterobacter cloacae* or *Citrobacter freundii* (10^7 cfu/ml) in diffusion chambers (75) that were immersed in Bozeman chlorinated drinking water as previously published (17,75-78,122,123). Injury was determined as the percent difference between the number of colony forming units on the nonselective medium (TLY) and the selective medium (TLY-D) (78). The recovery of injured coliforms was evaluated in the presence of the following selective agents: Tergitol 7 (Baker), Tergitol 4, Tergitol 8, polyoxyethylene ether W-1, and Triton X-100 (all from Sigma) in PLY agar (proteose peptone #3, 5.0 g, lactose 20 g, yeast extract 3.0 g and 15 g agar per liter distilled water, see Table 3). Differential agents that were tested included: brom thymol blue (sodium salt, Sigma), brom cresol purple (Difco), eosine B, eosine Y, methylene blue, aniline blue, janus green (all from Baker) and were added to PLY agar. In all cases coliforms were enumerated by the spread plate technique and incubated at 35°C for 24 h (2).

Media preparation and microbiological analysis

The formulation and preparation instructions for m-T7 agar are shown in Table 3. Occasionally high numbers of Gram positive bacteria in drinking water (62) produced a false positive reaction. Additional selectivity was obtained by aseptically adding penicillin G (0.1 ug/ml, Sigma) to the medium after autoclaving. Stock solutions of 0.01 mg/ml penicillin G were filter sterilized using a 0.22 um membrane filter (Millipore Corp) and frozen in 2.5 ml amounts. When m-T7 medium was prepared, one 2.5 ml vial of the antibiotic was added to 250 ml of tempered agar. Penicillin G may be stored frozen for 6 months, and prepared plates may be stored at refrigerator temperature for up to one week provided excessive drying does not occur (2,38,100).

Media performance was evaluated by filtering three replicates of each sample dilution using six MF techniques: The standard and resuscitation MF techniques (labeled m-Endo and m-Endo + LTB, respectively) were conducted according to established procedures using Millipore HC type filters, m-Endo agar and lauryl tryptose broth (LTB) (both from Difco). Millipore HC type membrane filters were used because the surface pore size (2.4 um) has been indicated as optimum for recovery of injured fecal coliforms (78,108). In the third MF technique, plates prepared with approximately 7 ml of m-Endo agar were overlayed with 3 ml lactose agar (Difco) immediately prior to use (labeled m-Endo + LA). A single step and resuscitation MF procedure (labeled m-T7

Table 3. Formulation of m-T7 medium

Ingredient[a]	per liter distilled water
Proteose peptone #3	5 g
Yeast extract	3 g
Lactose	20 g
Tergitol 7 (25% solution)	.4 ml
Polyoxyethylene ether W1	5 g
Brom thymol blue	0.1 g
Brom cresol purple	0.1 g
Agar	15 g
Final pH[b]	7.4

[a] All ingredients manufactured by Difco Laboratories except polyoxyethylene ether W1 and brom cresol purple which were manufactured by Sigma Chemicals and tergitol 7 which was obtained from Baker Chemical Company.

[b] The medium was autoclaved at 121°C for 15 min and final pH was adjusted aseptically to 7.4 with 0.1 N NaOH.

Additional selectivity may be obtained by aseptically adding 0.1 ug/ml penicillin G to the medium after autoclaving. Media prepared with penicillin G should be used within one week wnen stored at 4°C.

and m-T7 + PLY, respectively) were developed using m-T7 agar. PLY broth rather than LTB was used to saturate sterile resuscitation pads (Millipore Corp.) on which membrane filters were preincubated. The last MF technique compared in this study used anaerobic incubation of m-T7 agar plates (labeled m-T7 A) in a BBL gaspak anaerobe jar. Comparisons between m-T7 agar and Tergitol 7 agar (Difco) were also made. Volumes sampled were 100 ml for drinking water, 0.1 ml for surface water, and 0.1-50 ml for laboratory chlorinated water samples. Because widely varying dilutions were used for different water samples, counts are tabulated on a per filter basis and are represented irrespective of dilution.

All smooth yellow convex colonies on m-T7 agar and typical green sheen colonies on m-Endo agar were counted with the aid of a dissecting microscope (15-20 x). At least 20% of the colonies were transferred from the MF into tubes of LTB for determination of gas production. Positive LTB tubes were not routinely transferred to brilliant green bile broth because of the inhibitory nature of this medium (27,28), but all gas producing isolates from m-T7 agar were identified using the API-20E system (Analytab Products, Plainview, NY).

Quality control and statistical comparisons

A quality assurance program as outlined in Standard Methods (2) and Microbial Methods for Monitoring the Environment (10) was used throughout the course of this study. Performance of media and sterility controls were determined on a per lot or a per batch basis. Materials used during each experiment were checked for sterility. The temperatures of autoclaves and incubators were monitored on a per use basis.

Statistical comparisons were made using the paired t-test.

RESULTS

To ensure the growth of injured coliforms on m-T7 agar, various selective ingredients were examined to determine the optimum level which permitted high recoveries of stressed organisms. A variety of surface active agents were initially tested including: Tergitol 4, Tergitol 7, Tergitol 8, polyoxyethylene ether W-1, Triton x-100 and Tween 80 (Table 4 part 1). Optimum concentrations were determined as the highest level of surface active agent which permitted 90-100% recovery of injured cells. The optimum concentration of Triton x-100 was 0.001%; Tween 80, 0.1%, Tergitol 7, 0.01%. Tergitol 4 was very inhibitory to injured cells even at the 0.001% level, while Tergitol 8 and polyoxyethylene ether W-1 were not inhibitory at the concentrations tested.

The selectivity of the surfactants at concentrations determined to be "non-injurious" to coliforms was evaluated by determining the percent inhibition of standard plate count bacteria

(SPC) from contaminated river water. Tergitol 7 inhibited 55% of SPC bacteria; polyoxyethylene ether W-1, 54%; Tergitol 8, 7%; Triton x-100, 7% and Tween 80 showed no inhibition (Table 4). A combination of Tergitol 7 and polyoxyethylene ether W-1 exhibited a 55% inhibition of SPC bacteria, while m-Endo agar showed 78% inhibition.

A variety of differential agents were evaluated as indicators of lactose fermentation (Table 4 part II). Compounds were either non-injurious and poor in differentiating lactose fermentation (eosin B), or fair to good in differentiating lactose fermentation but very inhibitory to injured coliforms (eosin Y, erythrosin, methylene blue, aniline blue, janus green). Brom thymol blue and brom cresol purple were not inhibitory to injured coliforms at the 0.01% level and gave good differential reactions when used in combination. In addition, the combination of the two dyes provided additional inhibition of non-coliform bacteria (Table 4).

Occasionally strains of *Staphylococcus* and *Micrococcus* produced false positive coliform reactions on m-T7 agar. Incorporation of 0.1 ug/ml (final concentration) penicillin G aseptically added to the medium after autoclaving prevented the growth of these organisms. Parallel tests of m-T7 agar with and without penicillin G gave equivalent recovery of both laboratory injured coliforms and coliforms from 9 surface and drinking water samples (data not shown).

To evaluate the efficiency of m-T7 agar for recovery of injured coliforms, cultures of *E. coli*, *K. pneumoniae*, *E. cloacae*, *C. freundii* were stressed (greater than 90% injury) in drinking water. Depending on the organism tested, m-T7 agar recovered 86-99% more coliforms than m-Endo agar (data not shown). In addition, 67 water samples were analyzed using m-T7 and m-Endo agars by various MF techniques. Overall, m-T7 agar recovered significantly more ($p < 0.05$) coliforms than either the standard m-Endo or the m-Endo with LTB resuscitation techniques (Figures 4 and 5). For all waters tested, m-T7 agar recovered 43% more coliforms than m-Endo agar and 36% more coliforms than m-Endo agar with LTB resuscitation. In one instance, m-T7 agar recovered 17 confirmed coliforms, while m-Endo agar recovered none (Figures 4 and 5). From the 44 drinking water samples analyzed, m-T7 agar recovered 2.7 times more coliforms than the m-Endo resuscitation technique and nearly 3 times more coliforms than the standard m-Endo technique (Table 5). From drinking water samples, m-T7 agar recovered more verified coliforms than any of the other five MF techniques (Table 5).

The single step m-T7 agar technique is the easiest method for analysis of drinking water, but other methods (m-T7 + PLY and m-T7 A) were used to compare the effectiveness of the single step technique. Addition of a resuscitation step to m-T7 agar generally did not increase overall coliform recoveries but the resuscitation technique did recover more coliforms than m-T7 agar from

Table 4. Effect of various indicator/selective agents on the inhibition of SPC bacteria and recovery of injured *E. coli*

Indicator/selective agent[a]		% of injured coliform recovered[b]	% SPC[c] inhibited
Part I Surfactants			
Tergitol 4	0.001%	85	ND[d]
	0.005%	85	ND
	0.01%	60	ND
Tergitol 7	0.01%	92	55
	0.05%	72	ND
	0.1%	33	ND
Tergitol 8	0.01%	105	ND
	0.05%	88	ND
	0.1%	95	7
Triton X-100	0.001%	100	7
	0.005%	73	ND
	0.01%	29	ND
Tween 80	0.1%	104	0
	0.5%	73	0
	1.0%	61	0
Polyoxyethylene ether W1	0.05%	95	51
	0.1%	100	51
	0.5%	102	54
Polyoxyethylene ether W1 (0.5%) + Tergitol 7 (0.01%)		97	55

(continued)

Table 4 (continued)

Indicator/selective agent[a]		% of injured coliform recovered[b]	% SPC[c] inhibited
Part II Indicators			
Brom thymol blue	0.01%	113	37
	0.05%	92	87
Brom Thymol blue (0.01%) + Brom cresol purple (0.01%)		99	58
Brom cresol purple	0.005%	114	32
	0.01%	103	23
	0.05%	90	75
Eosin B	0.001%	103	27
	0.005%	102	19
	0.01%	117	23
Eosin Y	0.001%	86	49
	0.005%	24	82
	0.01%	37	85
Erythrosin	0.001%	83	74
	0.005%	17	94
	0.01%	9	99
Methylene blue	0.001%	88	32
	0.005%	76	53
	0.01%	76	55
Aniline blue	0.001%	64	ND
	0.005%	25	ND
	0.01%	16	ND
Janus Green	0.01%	14	ND
m-Endo Agar		53	74

[a] The basal medium contained PLY agar

[b] Between 90 and 99% of the coliforms were injured

[c] The percent inhibition of SPC bacteria in Gallatin River water was calculated relative to the non-selective PLY counts.

[d] ND = not done

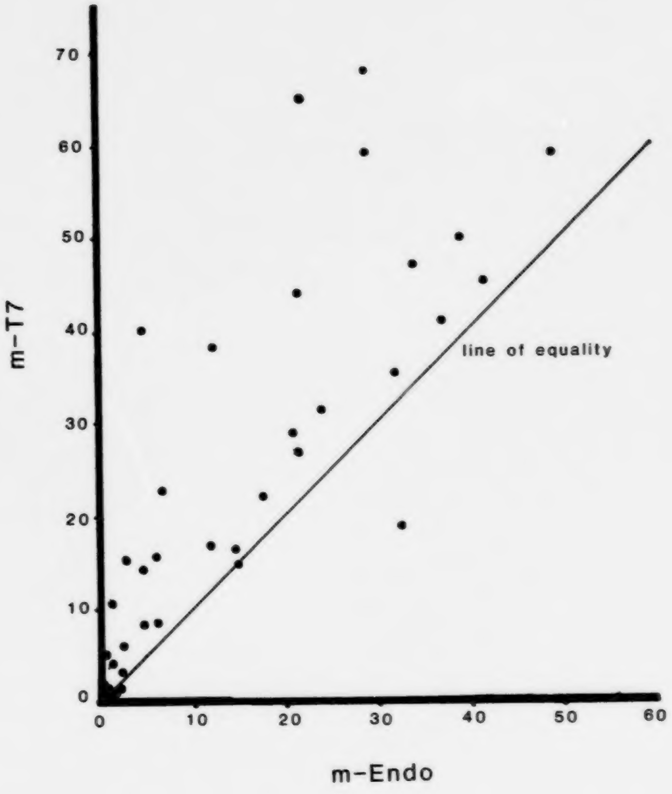

Figure 4. Comparison of coliform recoveries from water samples on m-Endo and m-T7 agar. Counts are represented as relative values.

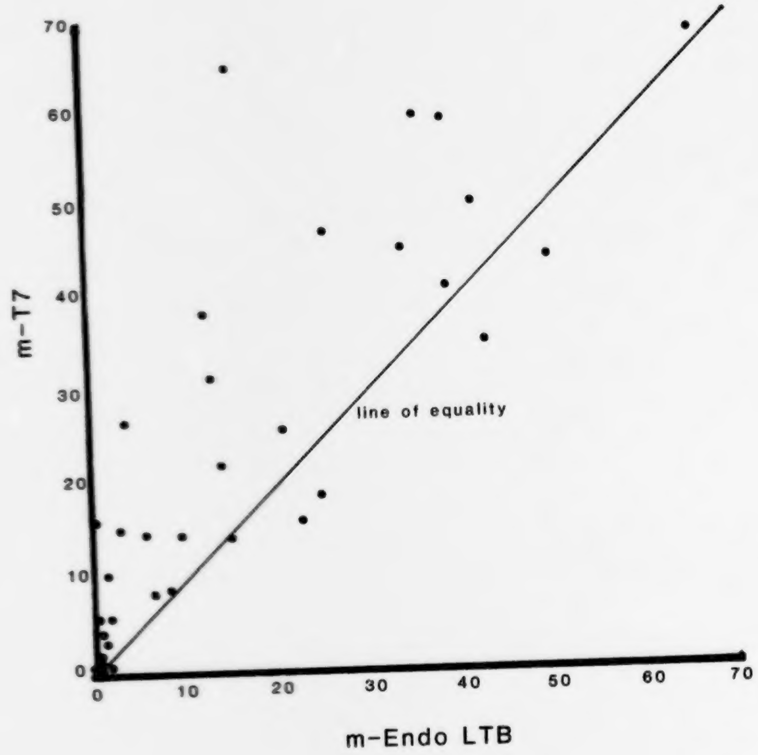

Figure 5. Comparison of coliform recoveries from water samples on m-Endo agar with lauryl tryptose resuscitation and m-T7 agar. Counts are represented as relative values.

laboratory-chlorinated surface water samples.

Anaerobic incubation of m-T7 agar plates was used as a means of inhibiting SPC bacteria, since the majority of SPC bacteria in drinking water are obligate aerobes (42,62). This technique was effective for surface water and chlorinated surface water where high densities of "background" bacteria existed (Table 5). However, anaerobic incubation of m-T7 agar proved to be inadequate in recovering more coliforms from drinking water samples than the standard m-Endo agar technique. A lactose agar overlay method was used as another way of resuscitating injured coliforms since previous research has indicated that LTB may inhibit as many as 79% of injured coliform organisms (78). The overlay technique recovered significantly more ($p < 0.05$) coliforms than the LTB resuscitation method when only chlorinated surface water samples were tested. For surface water and drinking water there was no statistical difference in coliform recoveries between the methods.

Initial studies compared coliform recoveries of Tergitol 7 agar with m-T7 agar. m-T7 agar recovered 2.2 times more coliforms from 19 surface and drinking water samples than Tergitol 7 agar (9.69 and 4.45 respectively). Moreover, only 49.7% of the 169 presumptive coliform isolates on Tergitol 7 agar produced gas in LTB. Because of low coliform recoveries and low verification rates further evaluation of Tergitol 7 agar was not continued.

Nearly 240 non-yellow, background colonies from m-T7 agar were tested for false-negative coliform reactions. These colonies were inoculated into LTB and tested for gas production after 48 h at 35°C incubation. Less than 0.5% (1 coliform) proved to be a false-negative from m-T7 agar. This organism was identified as _Serratia rubidaea_ by the API 20E system. In addition, up to 10 coliform colonies from each technique and sample were verified for gas production by inoculating tubes of LTB. Over 930 yellow coliform colonies from m-T7 agar were checked for gas production; of these 658 (70.6%) were positive. Colonies picked from m-Endo agar had a confirmation rate slightly lower than m-T7 agar; 69.6% of the 860 coliform colonies tested produced gas in LTB.

A total of 295 coliforms isolated on m-T7 agar have been identified by the API 20E system (Table 6). The predominant genera were _Escherichia_ (17.6%), _Klebsiella_ (21.7%), _Enterobacter_ (32.2%) and _Citrobacter_ (17.3%). About 5% of the isolates were _Aeromonas_, _Serratia_ and _Proteus_ and 7.5% were not identified. Over 90% of the coliforms isolated on m-T7 agar gave typical green-sheen colonial reactions when restreaked on m-Endo agar.

DISCUSSION

The many problems associated with current m-Endo type media formulations prompted us to develop a new medium capable of accurately enumerating coliform densities from drinking water. One of the most important considerations is the inhibitory nature

Table 5. Comparison of m-Endo and m-T7 Techniques.

	Relative Coliform Counts		
Medium	Drinking Water n = 44[a]	Surface Water n = 11	Chlorinated Surface Water n = 12
m-Endo	1.41	27.8	13.6
m-Endo + LTB pad[b]	1.48	32.3*[c]	14.2
m-Endo + LA overlay[d]	1.27	32.8*	22.6*
m-T7	3.96*	39.9*	20.6*
m-T7 + PLY pad[e]	3.85*	40.3*	25.2*
m-T7 - anaerobic	0.57	37.4*	22.8*

[a] n; number of samples

[b] LTB pad; lauryl tryptose broth pad

[c] * indicates a significantly greater value compared to m-Endo ($p < .05$)

[d] LA overlay; lactose agar overlay

[e] PLY pad; base composition of m-T7 containing proteose peptone #3, lactose and yeast extract.

Table 6. Identification of Coliforms Isolated on m-T7 Agar.

Organisms	# of times isolated[a]	%
Escherichia coli	42	17.6
Enterobacter agglomerans	46	15.6
Klebsiella pneumoniae	64	21.7
Citrobacter freundii	51	17.3
Enterobacter aerogenes	7	2.4
Enterobacter cloacae	42	14.2
Aeromonas spp.	7	2.4
Serratia rubidaea	1	.3
Serratia liquefaciens	2	.7
Proteus morganii	1	.3
not identified	22	7.5
Total	295	100.0

[a] Coliforms were isolated from surface water, chlorinated surface water and drinking water (see Materials and Methods).

of the selective ingredients in m-Endo type agar. Coliforms in the environment may be stressed by exposure to a variety of factors including: chlorine and other disinfectants, heat, freezing, acid mine drainage, transition metals, sunlight and ultraviolet light (6,15,17,37,53). Previous research has shown that various media formulations commonly used for water analysis containing more than 0.05% bile salts or desoxycholate were highly inhibitory to injured coliforms (78). m-Endo agar contains 0.1% desoxycholate plus 0.005% sodium lauryl sulfate and has been shown to inhibit as many as 70% of the injured coliforms (78). Because of inadequate techniques to measure injury, the extent of injured coliforms in drinking water is largely unknown, although some reports estimate that coliforms in aquatic environments are recovered with efficiencies of 10% or less (17,29,75,76). The results of this study indicated that two-thirds of the coliforms present in drinking water samples were injured.

The second problem associated with m-Endo-type agar formulations is the inability to distinguish coliforms from non-coliforms. Coliforms are differentiated from other bacteria on m-Endo-type media by the production of a dark colony with a metallic green sheen (2). In one study, nearly 25% of the non-sheen background colonies on m-Endo LES agar produced gas in m-LAC broth and were identified as *Citrobacter*, *Enterobacter*, *Escherichia* or *Klebsiella* (35). Such occurrences of false negative coliforms on m-Endo agar are particularly distressing since these organisms would not be interpreted as an indication of potentially contaminated drinking water. Several outbreaks of *Salmonella* and Poliomyelitis virus have been reported in drinking water which had no or low coliform levels, possibly because injured coliforms did not grow on m-Endo agar or because the indicator organisms failed to produce a typical green-sheen colony (11,70,82,105).

The accuracy and sensitivity of the MF technique are greatly influenced by the efficiency of the verification procedure employed. As many as 56% of the typical green sheen colonies on m-Endo agar may not produce gas in lactose broth (29,35,41). These false-positive reactions on m-Endo-type agar have been attributed to sheen production by slow lactose fermenters (41), Gram-positive bacteria (41) and synergistic reactions producing a sheen by two non-coliforms (102). Recently however, methods used for the confirmation of gas production have been attributed to cause low verification rates (35,79). Factors known to influence the rate and amount of gas production in lactose-containing media include: nutritional composition (18,22), amount of buffer (18,41), media volume and Durham tube size (38).

An additional problem with m-Endo-type media is the availability and quality of basic fuchsin (Geldreich, personal communication). Reproducibility of water sampling data depends heavily on the consistent quality and adequate supply of all ingredients in the specified media formulations. Recently, changes in quality and solubility of dyes in m-Endo-type agar have been noted.

It is unknown at this time how much effect dye quality has on coliform detection.

The results of this report have demonstrated that there is an effective alternative medium to m-Endo agar for the analysis of coliforms in drinking water. Steps taken in the formulation of m-T7 agar have ensured that the medium is selective while minimizing the inhibition of stressed coliforms. The effectiveness of m-T7 agar is evidenced by a 43% overall increase in verified coliform counts from all samples tested (Figure 4). High recoveries of laboratory injured coliforms as well as a three-fold increase in coliform recoveries from drinking water samples were also observed (Table 5). In addition to yielding high coliform counts, m-T7 agar also had a false-negative rate less than 0.5%. Only one non-yellow colony out of 239 tested proved to be a coliform.

In this study both m-Endo and m-T7 agar had high rates of false positive coliforms. However, preliminary data of on-going studies indicate that of the 30% coliform colonies not producing gas in lauryl tryptose broth, nearly 80% of the 145 isolates tested were o-nitrophenyl-B-D-galactopyranoside (ONPG) positive and cytochrome oxidase negative. At present, 29 of these organisms have been identified, 66% were E. agglomerans; 17%, E. coli; 10%, K. pneumoniae; 3%, C. freundii; and 3% E. cloacae. It is possible that yellow pigmented bacteria may occasionally be counted as false-positive coliforms, but with experience pigmented organisms and coliforms producing a yellow acid reaction on m-T7 agar can easily be distinguished.

There was no problem with excessive background colonies on m-T7 agar with the 44 drinking water samples examined in this study. However, there was some crowding of colonies on the filters of both types of media when surface water samples containing sewage effluent were examined. The data in Table 5 indicates that the single step MF technique may be used with m-T7 agar for analysis of coliforms in all waters, but anaerobic incubation may facilitate the recovery of coliforms from highly contaminated surface waters.

Most of the coliforms isolated on m-T7 agar were identified as members of one of the four commonly accepted coliform genera (Table 6). It is not possible to determine from these data if m-T7 agar was biased in the types of coliforms detected. However, over 90% of the coliforms isolated on m-T7 agar gave typical green-sheen reactions when restreaked on m-Endo agar. It can be concluded that the difference in recoveries between the two media is due to injured coliforms not capable of growing on m-Endo agar and not due to atypical or unusual coliform isolates.

While this report has demonstrated the superiority of m-T7 agar over m-Endo agar in the recovery of coliforms from Montana drinking water, additional testing needs to be done to fully evaluate the effectiveness of m-T7 agar in other regions. In addition, other problems associated with the MF technique such as efficiency of verification procedures, effects of particulates and SPC organisms on recovery of indicator bacteria, and factors relating to aquatic injury require further investigation to make the indicator concept a more reliable predictor of potable water quality.

SECTION 3

EVALUATION OF m-T7 AGAR AS A FECAL COLIFORM MEDIUM

SUMMARY

m-T7 agar, designed to improve recoveries of injured total coliforms was evaluated for its effectiveness as a fecal coliform medium. Although the data presented are limited and therefore the results are preliminary, the time and temperature of preincubation was found to be crucial to the optimum recovery of fecal coliforms. Isolation rates for fecal coliforms on m-T7 agar were the highest when plates were preincubated 8 h at 35-37°C before transfer to 44.5°C for 12 h. The medium was found to produce consistently higher fecal coliform counts than all the other methods tested. Recoveries were 5.6 times greater than the standard m-FC method and 1.5 times greater than the two layer enrichment, temperature acclimation procedure. Verification rates for fecal coliforms isolated on m-T7 agar averaged 91.2% while verification rates for m-FC agar averaged only 83.9%. Both media isolated similar fecal coliform populations. The advantages of a single medium, highly effective for the isolation of both total and fecal coliforms are discussed.

INTRODUCTION

It is well recognized that coliforms in chlorinated sewage effluents may be stressed or injured and may not grow when enumerated on selective media (7,12,17,67). Lin (67), in 1973, observed fecal coliform levels from chlorinated sewage effluents enumerated by the membrane filter (MF) technique using m-FC medium at 44.5°C were lower than fecal coliform recoveries by the elevated temperature multiple tube procedure (MPN). Braswell and Hoadley (12) reported that *Escherichia coli* injured during chlorination of secondary sewage failed to produce colonies on m-FC agar or grow and produce gas in lactose broth.

Under non-restrictive conditions injured coliforms can regain the ability to grow on selective media through a resuscitation process (7,17). As a result, various resuscitation techniques have been proposed (44,68,91,99,110). Rose, et al. (99) reported an improved fecal coliform method using m-FC agar with a lactose broth agar overlay. Plates were preincubated at 35°C for 2 h, at which time the temperature was increased to 44.5°C for 22-24 h. When tested with a variety of water samples, the two layer method recovered almost twice the number of fecal coliforms compared to the standard MF technique. In 1976, Lin (68) proposed a similar resuscitation method using phenol red lactose broth and incubation at 35°C for 4 h followed by plating on m-FC agar at 44.5°C for an additional 18 h. This two step MF technique produced fecal coliform counts comparable to the MPN procedure. Other investigators have reported increased fecal coliform recoveries by eliminating rosolic acid from the medium formulation or by simply preincubating m-FC agar plates for 5 h at 35°C before shifting to 44.5°C for 18 h (44,68). Based on a physiological approach, Stuart, et al. (110) developed an injury-mitigating MF (IM-MF) procedure that included glycerol and acetate plus reducing agents in both layers of a two layer medium. Fecal coliform recoveries were 1.3 to 19 times greater than the standard m-FC recoveries.

In recognition of the problems of enumerating injured bacteria, a new section entitled "Stressed Organisms" (section no. 920) was added to the 15th edition of _Standard Methods for the Examination of Water and Wastewater_ (2).

Recently, we developed a new medium (m-T7 agar) for the improved recovery of injured total coliforms (64). We observed that aniline blue and bile salts, two ingredients in m-FC agar, were very inhibitory to injured coliforms (64,78). m-T7 agar contains neither of these ingredients and has been shown to be superior to m-Endo agar for the recovery of total coliforms from drinking water. The present study was designed to determine if m-T7 agar was effective for recovery of injured fecal coliforms as well.

MATERIALS AND METHODS

Sample area and sample collection

Secondary sewage effluents were collected from the chlorinated wet well, contact chamber and out-fall ditch of the sewage treatment plant in Bozeman, MT., during the mid-morning hours (9-11 a.m.) of October, 1982, through January, 1983. The sewage treatment plant receives domestic and light industrial waste as well as runoff from a stormwater collection system. Effluent free residual chlorine levels ranged from 0 to 0.6 mg/l (average 0.24 mg/l) and total chlorine levels ranged from 0.14 to 0.7 mg/l (average 0.34 mg/l). Effluent temperatures ranged from 9 to 16°C (average 10.8°C) and pH ranged from 6.3 to 7.5 (average 7.0).

Samples were collected in sterile 2 l polypropylene bottles containing 0.008% (final concentration) sodium thiosulfate (2). Chlorine residuals were determined on site by the N,N,diethyl-p-phenylene diamine (DPD) colorimetric method using a Hach model CN-70 chlorine test kit (Hach Chemical Company, Loveland, CO). Samples were placed on ice and transported to the laboratory within 1 h. A preliminary test was done for some samples to determine the approximate fecal coliform level. In these cases, the samples were stored at 4°C overnight. All samples were analyzed within 30 h after collection.

Microbiological Analyses

Media comparisons were made by filtering three replicates of each sample dilution through Millipore HC type filters (Millipore Corp., Bedford, MA) using the following six MF techniques. The standard fecal coliform procedure and two resuscitation techniques were performed according to Standard Methods (sections 909C and 920) (2). One resuscitation technique consisted of eliminating rosolic acid from the m-FC medium. The other resuscitation method was similar to the two layer enrichment, temperature acclimation procedure of Rose, et al. (78). These three techniques were labeled m-FC S (standard), m-FC R^- (without rosolic acid) and m-FC LA (m-FC agar with a lauryl tryptose agar overlay; incubation at 35°C for 4 h followed by 20 h at 44.5°C). m-T7 agar (64) was used as the fecal coliform medium in the next three techniques. One method consisted of simply incubating the medium at 44.5°C for 24 h (labeled m-T7 44.5°C). The other two techniques involved preincubating the plates for 8 h at 35°C (labeled m-T7 8 h, 35°C) or 37°C (labeled m-T7 8 h, 37°C) followed incubation at 44.5°C for an additional 12 h.

All smooth yellow convex colonies on m-T7 agar or blue colonies on m-FC agar were counted with the aid of a dissecting microscope (15-20x). At least 10 presumptive fecal coliforms from each technique and sample were verified by inoculation into lauryl tryptose broth (35°C for 48 h) followed by transfer to EC broth (44.5°C for 24 h). Fecal coliforms were identified using the API-20E system (Analytab Products, Plainview, NY).

Media and Materials

m-FC broth base (lot no. 689377) supplemented with 1.5% agar (lot no. 686162 and 704723), lauryl tryptose broth (lot no. 685074), and EC broth (lot no. 701122) were purchased from Difco Laboratories, Detroit, MI. Rosolic acid (lot no. 81F-007) was obtained from Sigma Chemical Co., St. Louis, MO. m-T7 agar (containing 0.1 ug/ml penicillin G) was prepared as previously published (64). A heat sink incubator and a circulating water bath both capable of achieving 44.5 ± 0.2°C were used for the elevated temperature tests.

Quality control and statistical comparisons

A quality assurance program as outlined in Standard Methods (2) was used throughout the course of this study. Performance of media and sterility controls were determined on a per lot or a per batch basis. Materials used during each experiment were checked for sterility. The temperatures of autoclaves and incubators were monitored on a per use basis.

Because widely varying dilutions (1 ml - 10^{-3} ml) were used for different water samples, counts are tabulated on a per filter basis and are represented irrespective of dilution. Statistical comparisons were made using the paired t-test.

RESULTS

Temperature has been recognized as a major selective factor in the fecal coliform procedure. As a result, experiments were designed to determine the optimum preincubation time and temperature for m-T7 agar. Plates were preincubated at 35°C for up to 8 h before transfer to 44.5° (total incubation time for all plates was 24 h) (Figure 6). Results indicated only a small increase in fecal coliform recoveries during the first 4 h, but a dramatic increase (3.43 fold) in fecal coliform recoveries was observed with a preincubation time of 8 h. Similar increases were seen for m-FC agar, but after 4 h at 35 °C many small blue non-coliform background colonies also appeared. These background colonies were not seen on m-T7 agar and generally were not a problem.

In order to determine the optimum temperature, m-T7 agar plates were preincubated for 8 h at various temperatures including 25°, 32°C, 35°C and 37°C (Figure 7). Highest fecal coliform recoveries were observed by preincubating m-T7 agar plates at 37°C for 8 h. Isolation rates for fecal coliforms at 37°C (8 h) were 32% higher than fecal coliform recoveries at 35°C or 32°C, 68% higher than recovery rates at 25°C and nearly 10 times the isolation rates for fecal coliforms without any preincubation (Figure 7). Combinations of incubation times and temperatures (example; 25°C for 4 h, then 35°C for 4 h followed by 16 h at 44.5°C) did not improve fecal coliform recoveries above the 37°C (8 h) level. Verification of fecal coliforms isolated by this techinque (37°C for 8 h preincubation time) yielded a 98% verification rate.

Repeated analyses indicated that fecal coliform counts did not significantly increase after 20 h total incubation time (data not shown), rather colonies on m-T7 agar incubated for a full 24 h produced so much acid that neighboring non-fecal coliform colonies started to take on a yellowish appearance. The difference between fecal and non-fecal coliform colonies was much more distinct when plates were incubated for a total time of 20 h.

Fecal coliform counts for six techniques tested on eleven samples of secondary sewage effluent are given in Table 7. Recovery of fecal coliforms by the m-T7 8 h, 35°C technique was consistently higher than either the m-FC S or m-FC R$^-$ procedures. Eighty percent of the time the m-T7 8 h, 35°C technique exceeded the m-FC LA method. Statistical analyses showed that the m-T7 8 h, 35°C technique was significantly higher ($p < 0.001$) than all three m-FC methods. The m-T7 8 h 37°C procedure was consistently higher than all the other methods for the five samples tested (Table 7). Recoveries were an average of 5.6 times greater than the standard m-FC method and 1.5 times greater than the m-FC LA procedure. Where parallel analyses of m-T7 8 h, 35°C and m-T7 8 h 37°C were done, m-T7 8 h 37°C resulted in a 26% increase in fecal coliform recovery over the m-T7 8 h 35°C method. However, because few comparisons were made this difference was not con-

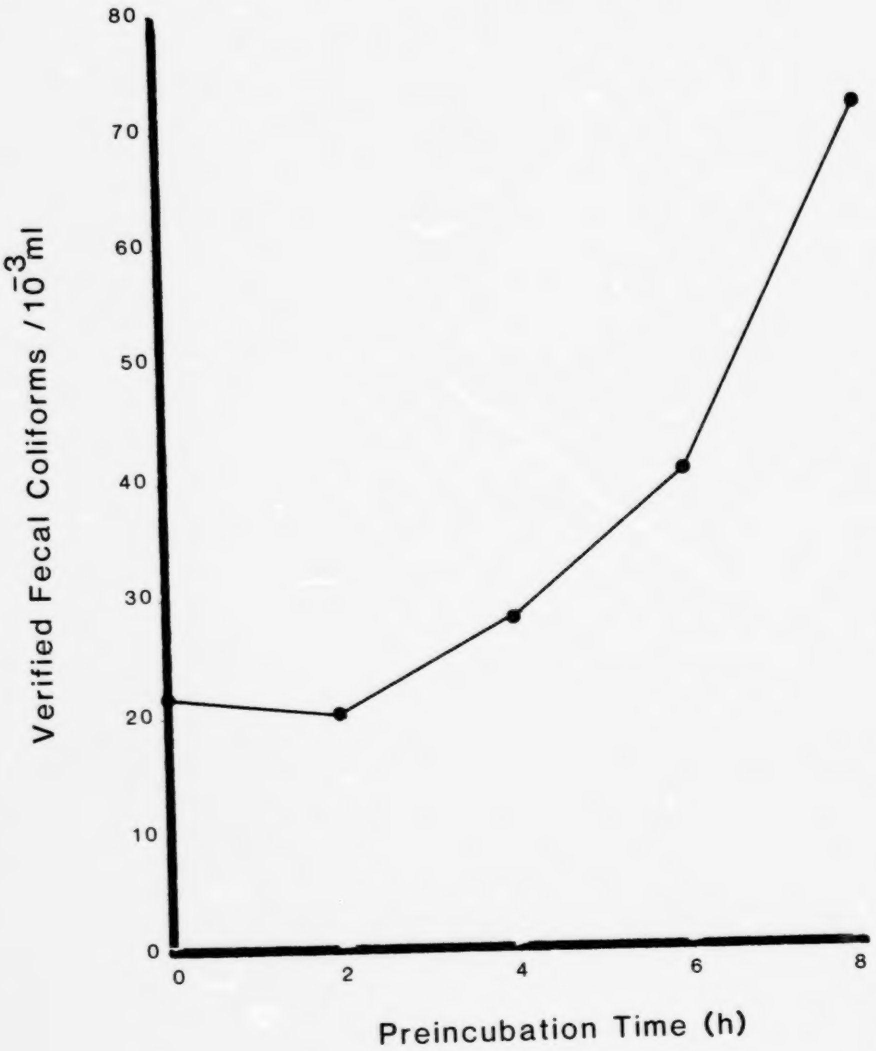

Figure 6. Effect of preincubation time on fecal coliform recovery for m-T7 agar. Plates were incubated up to 8 h at 35°C. Total incubation time for all plates was 24 h.

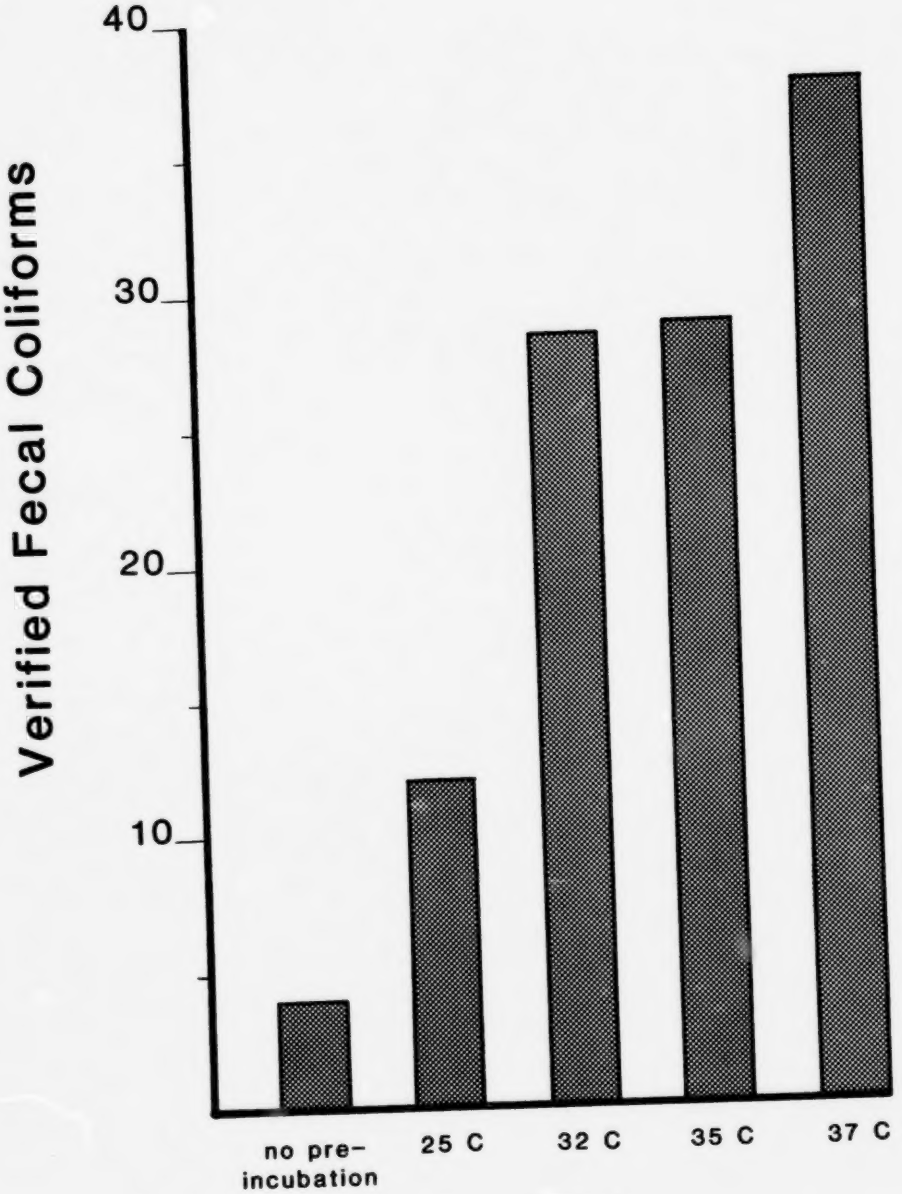

Figure 7. Comparison of preincubation temperature for fecal coliform recovery on m-T7 agar. Plates were incubated at the designated temperatures for 8 h followed by 16 h at 44.5°C.

Table 7. Comparison of m-FC and m-T7 techniques.

Date	Site	pH	Temperature (^oC)	Free Chlorine (mg/l)	Total Chlorine (mg/l)	Dilution Factor (per 100ml)	m-T7 44.5^oC	m-FC S	m-FC R$^-$	m-FC LA	m-T7 8 h, 35^oC	m-T7 8 h, 37^oC
10/11/82	Outfall ditch	6.9	9.0	0.2	0.3	4.0×10^4	10.2	12.5	14.4	20.3	27.8	ND[a]
10/11/82	Wet well	6.7	10.0	0.6	0.7	4.0×10^4	5.1	11.2	9.0	15.7	18.4	ND
10/18/82	Outfall ditch	7.2	11.0	0.2	0.3	4.0×10^4	12.6	13.6	25.6	38.7	36.0	ND
10/18/82	Wet well	7.2	10.0	0.2	0.5	4.0×10^4	16.0	22.5	29.0	37.3	80.6	ND
10/25/82	Outfall ditch	7.5	11.5	0.2	0.2	4.0×10^4	5.1	7.6	12.8	46.0	83.3	ND
10/25/82	Wet well	7.5	11.0	0.2	0.2	4.0×10^4	12.0	14.5	23.7	24.7	53.9	ND
12/6/82	Contact chamber	7.2	16.0	0.3	0.5	5.0×10^3	12.6	8.5	24.3	55.2	ND	70.5
12/13/82	Contact chamber	7.2	11.0	0.2	0.2	5.0×10^3	3.9	5.6	8.1	10.8	16.8	24.6
1/3/83	Contact chamber	7.1	9.0	0.0	0.1	1.0×10^3	6.0	17.1	17.4	21.6	40.3	47.0
1/10/83	Contact chamber	6.3	11.0	0.1	0.3	6.7×10^1	8.6	7.2	16.8	33.0	30.1	39.0
1/24/83	Contact chamber	6.9	9.0	0.4	0.5	1.6×10^1	2.0	6.7	7.4	22.6	23.0	28.3

a) See Materials and Methods for full description of procedures. Abbreviation: ND = not done.

sidered statistically significant ($p = 0.10$).

The mean values for five of the six techniques are represented in Figure 8. Overall, m-T7 8 h 35°C yielded greater recoveries of fecal coliforms than any of the m-FC procedures. m-T7 8 h 35°C showed a 3.53 fold increase over the standard m-FC technique and a 1.51 fold increase over the two layer enrichment, temperature acclimation procedure (m-FC LA). Verification rates for the three m-T7 agar techniques were 94% (103/110) for m-T7 44.5°C, 85% (85/100) for m-T7 8 h, 35°C and 98% (49/50) for m-T7 8 h, 37°C (overall average, 91.2%). Slightly lower verification rates were observed for the m-FC procedures; 74% (82/110) for m-FC S, 87% (96/110) for m-FC R^- and 90% (99/110) for m-FC LA (overall average, 83.9%).

Identification of 60 fecal coliforms isolated by the m-FC LA and m-T7 8 h, 35°C procedures revealed that the two media recovered nearly the same coliform populations. Escherichia coli was the predominant fecal coliform on both media comprising 70% of the isolates on m-T7 agar and 63.3% of the isolates on m-FC medium. Klebsiella pneumoniae totalled 26.7% and 33.3% of the isolates on m-T7 and m-FC agar, respectively. Enterobacter agglomerans was isolated once on m-T7 agar and Citrobacter freundii once on m-FC agar as fecal coliforms.

DISCUSSION

In this study, m-T7 agar preincubated at 37°C for 8 h before transfer to 44.5°C for 20 h was superior to all other methods tested. Although the data are limited and preliminary, this new technique recovered an average of 5.6 times more fecal coliforms than the standard m-FC method and 1.5 times greater than the m-FC LA procedure. The reason for the improved recovery is two fold: (i) use of a medium specifically designed to improve recoveries of injured coliforms, and (ii) adequate preincubation time at an optimum temperature to allow for complete recovery of stressed coliforms.

Prior to the development of m-T7 medium we surveyed over 20 different media commonly used for coliform analysis (78). The observation that the majority of selective media used to isolate Gram negative bacteria recovered 30% or less of the injured coliforms was related to the concentration of deoxycholate and bile salts. Currently, all media formulations for fecal coliform enumeration, except m-T7 agar, contain deoxycholate or bile salts as a selective ingredient (28,44,68,91,99,110). In addition, subsequent studies demonstrated that aniline blue, the differential agent in m-FC medium, was highly toxic to injured coliforms (64). By examining each ingredient in the m-T7 formulation for optimal recovery of laboratory injured cells we were assured of a medium highly efficient for the isolation and enumeration of coliform from environments where injury is an important consideration.

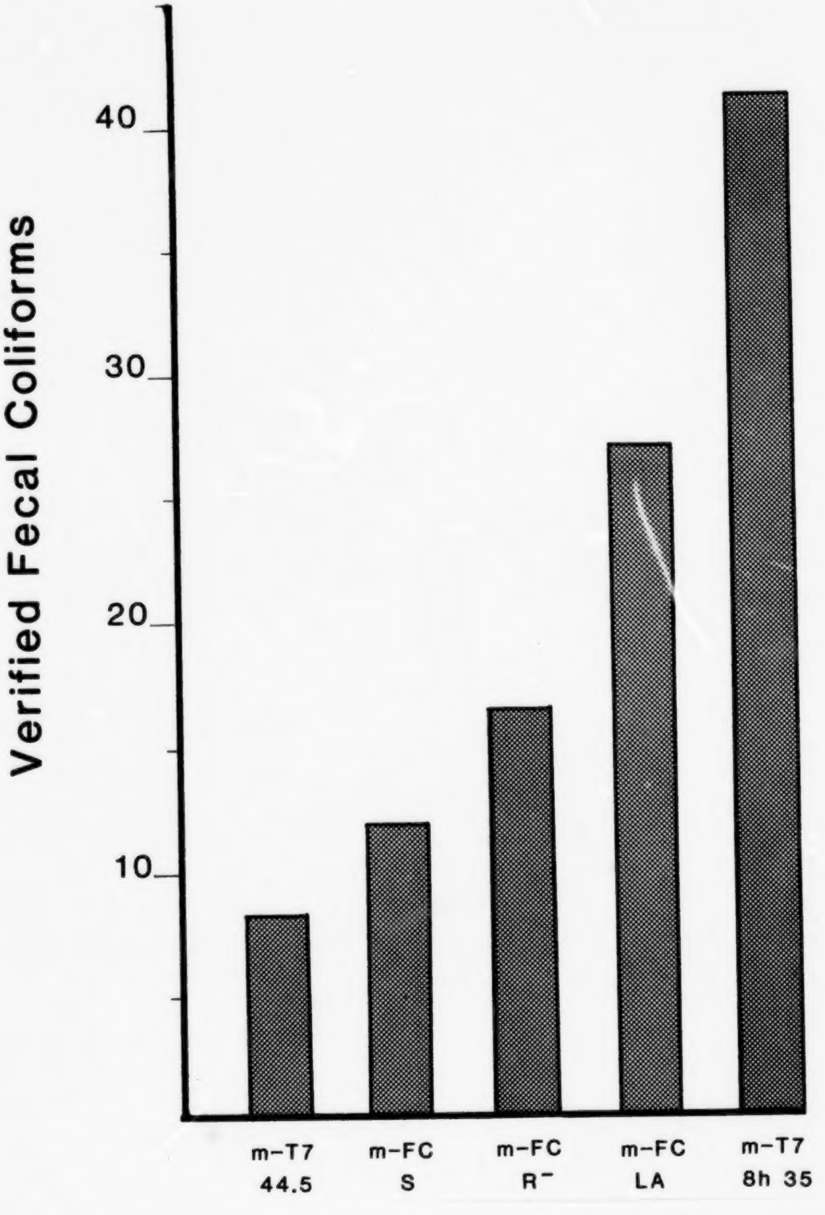

Figure 8. Comparison of fecal coliform recoveries from 10 chlorinated sewage effluent samples. See Material and Methods section for description of techniques. m-T7 8 h 37°C method is not compared because few parallel analyses were performed (see Table 7).

Results from this study have confirmed previous reports that temperature is a major inhibitory factor in the fecal coliform procedure when injured cells are being isolated. Accurate fecal coliform enumerations are not possible without adequate preincubation time to allow for complete resuscitation of stressed cells. Our data suggest that 8 hours at 37°C is necessary for optimal recovery of fecal coliforms. Using this preincubation time period significant increases in isolation rates were observed for both m-FC and m-T7 media. However, numerous small nonfecal coliforms were apparent on m-FC agar while these background organisms were not seen on m-T7 agar plates. The overall verification rate for m-T7 agar was 91.2% and the medium recovered the same fecal coliform population as the m-FC LA technique.

Results of this study indicated an average of 82.3% of the coliforms in chlorinated sewage effluents were injured (as determined by the difference between m-T7 8 h 37°C and m-FCS techniques). Previous laboratory studies have indicated the cell envelope as the major site of chlorine damage (17,123). Experiments have shown respiration, glucose transport and ATP levels all decrease in injured populations (17). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (123). These reports indicated repair of injured membranes usually occurred 3-4 h after resuscitation. In this study, optimal recovery of fecal coliforms from chlorinated sewage effluents did not occur until after an 8 h resuscitation period. It is unknown what physiological process requires an 8 h adaptation interval, but the data suggest the resuscitation time for bacteria injured in the environment may be longer than previously thought.

Several advantages are immediately apparent for having one medium capable of performing both the total and fecal coliform tests. (i) The medium is simple. There are no overlay procedures, nor does the filter need to be transferred. (ii) The medium is convenient. Since contamination of water supplies is usually transitory, it is neither expedient nor convenient to prepare another medium to assay for fecal pollution. In an emergency it would be easier to incubate the same medium at an elevated temperature for the fecal coliform test. (iii) Finally, use of one medium for two tests could save money. Costs could be cut by not having to purchase a variety of media and chemicals and by saving time in media preparation. One disadvantage is that an incubator is required capable of adjusting temperatures after an 8 h interval. Although the technology is readily available and a similar device is on the market (Millipore Corp., Bedford, MA), we are not aware of such an incubator designed specifically for this method. In addition, the proposed two-temperature fecal coliform test has a problem when successive sets of samples are processed during the day. However, such logistical problems can be overcome by several temperature-adjusting incubators. The initial cost of aquiring these incubators is well compensated for by the increased sensitivity and accuracy of the m-T7 8 h, 37°C technique.

SECTION 4

COMPARISON OF VERIFICATION PROCEDURES FOR THE MEMBRANE FILTER TOTAL COLIFORM TECHNIQUE

SUMMARY

Comparison of verification techniques for the membrane filter total coliform method were made using the standard lauryl tryptose broth (LTB) procedure and the B-galatosidase and cytochrome oxidase (B-gal/CO) method using coliforms isolated from 21 surface and drinking water samples. Of the 682 presumptive coliforms tested from all types of water samples, 577 (84.6%) verified using the B-gal/CO method while only 402 (58.7%) produced gas in LTB. When verification procedures included the confirmatory brilliant green-lactose-2% bile broth (BGLB) step, the verification rate dropped to 50.4%. Of the 146 presumptive coliforms tested from drinking water samples, 119 (81.5%) verified using the B-gal/CO method while only 15 (10.3% produced gas in LTB. Over 90% of the anaerogenic lactose fermenters verified by the B-gal/CO method were representative of typical coliform genera (Escherichia, Enterobacter, Klebsiella or Citrobacter). The data suggests that verification of total coliforms by standard procedures (LTB and BGLB) may result in significant underestimations of indicator densities particularly in waters where coliforms are stressed or injured.

The membrane filter (MF) techique is usually the method of choice for the analysis of total coliforms from potable water. The MF method is rapid, simple, and yields definitive results. However, factors such as elevated turbidity (36,40,63), injured coliforms (64,78), high numbers of non-coliform bacteria (21,40) and membrane filter type (78,108) may severely influence the sensitvity of the procedure. The U.S. Environmental Protection Agency has adopted a verification procedure because not all of the typical coliform colonies recovered by the MF technique will produce gas in lactose containing media (10,18,29,35,38,79). This scheme consists of subculturing typical coliform colonies into lauryl tryptose broth (LTB) and examining the culture tubes for gas production after incubation for 24 to 48 h (10,38). Further confirmation of coliform organisms is obtained by subculturing LTB positive tubes into brilliant green-lactose-bile broth (BGLB). The MF total coliform count is then adjusted by the percent of colonies verified as coliforms. The accuracy and sensitivity of the MF technique can therefore be greatly influenced by the efficiency of the verification procedure.

Evans et al. (35) reported significant variation in verification rates depending on the procedure used. Verification in m-LAC broth yielded a 50% increase in coliform counts compared to verification in LTB or lactose broth. Other investigators have shown that a buffered lactose containing medium increased gas production and reduced variability compared to unbuffered systems (79). The composition of lactose containing media has also been known to influence the rate and amount of gas produced by coliform bacteria (18,22).

Recently, an alternative verification procedure has been proposed (M. Pickett, T.J. Leahy, and W. Litsky, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981. p. 221; Q 124). In this method, presumptive coliforms were tested for the presence of B-galactosidase and cytochrome oxidase (B-gal/co). The developers of this scheme indicated that the enzymatic confirmation correlated more closely to the taxonomic definition of a coliform than did the standard verification procedure.

The present study was initiated when routine coliform analyses of drinking water samples yielded a high percentage of typical presumptive coliforms that did not produce gas in LTB.

Drinking water samples were collected from the distribution system of two small communities near Bozeman, MT. The first drinking water system serviced 2,000 residents and consisted of both surface and well water that was only intermittently chlorinated. The second system serviced 8,000 residents and consisted of a network of 8 unchlorinated wells. Raw water samples were collected from the East Gallatin River approximately one half mile (0.8 km) downstream from the outfall of a sewage treatment plant. Laboratory chlorinated samples were prepared in two ways: (i) surface water was treated with 1.0 mg/l free residual chlorine (avg. pH, 7.7; avg. temperature, 10°C), prepared daily from stock solutions of bleach (6% sodium hypochlorite by weight; Serco bleach distributed by S.E. Rykoff Co., Los Angeles, CA.); (ii) chlorinated distribution water (free residual chlorine varied from 0.5-1.2 mg/l chlorine pH 7.6, temperature 10°C) was mixed with 10% surface water. Both preparations were dechlorinated with sodium thiosulfate after 10 minutes contact (2).

Water samples were collected in sterile 2 l polypropylene containers with (drinking water) or without (surface water and samples for laboratory chlorination) added sodium thiosulfate (2). Samples were placed on ice and transported to the laboratory within 1 h after collection and analyzed within 5 h after collection.

Total coliforms were enumerated using two MF techniques. The standard MF technique was conducted according to standard procedures (2) using Millipore HA membranes (pore size 0.45 um) and m-Endo broth (Difco) supplemented with 1.5% agar (Difco). A new medium m-T7 agar, was also used since it was designed to improve recoveries of injured coliforms from drinking water (12).

All smooth, yellow, convex colonies on m-T7 agar and typical green sheen colonies on m-Endo agar were counted with the aid of a dissecting microscope (15-20x). At least 10 presumptive coliform colonies were picked from the MF and streaked for isolation on TSL agar (tryptic soy broth w/o dextrose, supplemented with 1.0% lactose and 1.5% agar; all from Difco). Plates were incubated 24 h at 35°C. Multiple colonies from TSL agar were inoculated into tubes of LTB (Difco). BGLB (Difco) tubes were inoculated from gas positive LTB tubes in some samples and incubated up to 48 h at 35°C. In addition, multiple colonies were transfered into tubes containing 1 ml of a 0.0166 M o-nitrophenyl B-D-galactopyranoside (ONPG, Sigma) solution (66) dissolved in phosphate buffer (2). ONPG tubes were incubated at 35°C for 4 h at which time 0.1 ml of 0.5 M Na_2CO_3 was added to enhance color production. Cytochrome oxidase test (66) was performed by placing few drops of a 1.0% solution of p-aminodimethylaniline oxalate on colonies grown on TSL agar. Oxidase positive organisms produced a deep purplish-blue color within 10 min. Coliforms were identified using the API 20E system (Analytab Products, Plainview, N.Y.).

A total of 682 presumptive coliform cultures were collected from 21 contaminated drinking and surface water samples. Of these presumptive positive isolates, 577 (84.6%) were verified as coliforms by the B-gal/CO method while only 402 (58.9%) were verified as coliforms by gas production in LTB broth (Table 8). When verification procedures included the BGLB step, the verification rate for liquid media dropped to 50.4%. For surface water and laboratory chlorinated surface water the B-gal/CO method verified 88.3% and 82.8%, respectively, of the presumptive coliforms while gas production in LTB occurred for 76.9% and 69.8% of the respective isolates. The most dramatic difference between the two verification techniques occurred with drinking water samples where the B-gal/CO method verified 81.5% of the 146 presumptive coliforms, while only 10.3% of the isolates produced gas in LTB (Table 8).

The inability of coliform isolates to produce gas was apparently not related to the presumptive isolation medium since 31.5% and 31.2% of the coliforms isolated on m-Endo and m-T7 agar, respectively, verified by the B-gal/CO method but were anaerogenic in LTB. All gas producing organisms identified as typical coliforms (*Escherichia*, *Klebsiella*, *Enterobacter* or *Citrobacter*) were also verified by the B-gal/CO method.

Identification of coliform isolates verified by the B-gal/CO method that were anaerogenic in LTB revealed that 91.5% of the organisms belonged to one of the four commonly accepted coliform genera (Table 9). *Enterobacter* *agglomerans* was the most common anaerogenic coliform (51.1%); while *Escherichia* *coli* was the second most common anaerogenic isolate (23.9%). *Aeromonas* was not observed as an anaerogenic isolate and comprised less than 3% of the total aerogenic coliform population.

Table 8. Comparison of verification procedures in drinking water, surface water, and chlorinated surface water for total coliforms.

Source	No. of samples	No. of presumptives	No. ONPG+ Co^-	% ONPG+ Co^-	No. gas+ in LTB	% gas+ in LTB
Drinking water	12	146	119	81.5	15	10.3
Chlorinated surface water	4	280	232	82.8	190	69.8
Surface water	5	256	226	88.3	197	76.9
Total	21	682	577	84.6	402	58.9

Abbreviations: ONPG, o-nitrophenyl-β-D-galactopyranoside; CO, cytochrome oxidase;

LTB, lauryl tryptose broth.

Table 9. Identification of anaerogenic lactose fermenters verified by the B-gal/CO method.

Organism	No. of times isolated	%
Klebsiella pneumoniae	5	10.6
Enterobacter agglomerans	24	51.6
Enterobacter cloacae	1	2.1
Citrobacter freundii	2	4.3
Escherichia coli	11	23.9
Yersinia enterocolitica	2	4.3
Serratia liquefaciens	1	2.1
Hafnia alvei	1	2.1
Totals	47	100

A total of 162 background bacteria (non-coliforms) from two surface water samples were examined by the B-gal/CO method. ONPG positive and cytochrome oxidase negative organisms not belonging to any of the coliform genera were frequently encountered (14.8%). These organisms were identified as *Pseudomonas maltophilia*, *P. cepacia*, *Staphylococcus sp.*, and *Bacillus sp.* These organisms may occur frequently in surface and distribution waters but rarely if ever yield typical presumptive coliform reactions.

A significant correlation existed between the level of injury (12,14) and the percent verification (using LTB) of coliforms from m-T7 agar ($r = ^{-}0.63$; $p < 0.001$). It is not known whether this correlation is related to some irreversible cellular injury which is involved in gas production from lactose, or reflects an association between anaerogenic coliforms and an increased susceptibility to aquatic injury.

In conclusion, the B-gal/CO method proved to be an accurate and sensitive verification procedure for total coliforms enumerated by the MF technique. Verification of coliforms by standard procedures (LTB and BGLB) may result in significant underestimations of indicator densities particularly in waters where coliforms may be stressed or injured.

SECTION 5

INVESTIGATION OF INTERACTIONS BETWEEN STANDARD PLATE COUNT BACTERIA AND COLIFORM ORGANISMS AND DEVELOPMENT OF A PRESERVATION TECHNIQUE FOR SAMPLE TRANSPORT AND STORAGE

SUMMARY

Preliminary studies were initiated to investigate the interactions between standard plate count (SPC) bacteria and coliform organisms, and to develop a preservation technique to eliminate these interactions during a 5 day transport/storage interval. Using spiked samples, it was shown that SPC bacteria could reduce coliform densities by more than 3 logs within 8 days. Some bacteria were able to cause injury in the coliform population. A significant correlation ($r = 0.66$; $p < 0.05$) was observed between the initial SPC level and the rate of coliform decline. Competition for limiting organic carbon was hypothesized to be a possible factor. A solution of sodium benzoate (10 g/l), chloramphenicol (32 ug/ml), EDTA (24 mg/l), yeast extract (1 gm/l), and peptone (1 g/l) was effective in preserving _Escherichia coli_ densities (80-100% viability) over a 5 day period. However, when this technique was used to preserve a contaminated surface water sample, extensive background growth occurred. Further research is proposed to more fully investigate nutrient competition and preservation techniques.

INTRODUCTION

Heterotrophic or "standard plate count" (SPC) bacteria in drinking water consist of a diverse group of microorganisms representing a wide range of metabolic activities, culture requirements and health concerns. Bacteria in water may be primary or opportunistic pathogens (4,14,66,92). These organisms could constitute a health risk to patients in hospitals, clinics, nurseries and rest homes. Standard plate count organisms in potable water may also create taste, odor or spoilage problems in products produced by food, beverage, cosmetic and drug industries (40,42). Victoreen (115) indicated that elevated levels of _Arthrobacter spp_. were responsible for taste and color problems in distribution waters. In addition, some species and strains of _Pseudomonas_, _Sarcina_, _Micrococcus_, _Flavobacterium_, _Proteus_, _Bacillus_, actinomycetes, and yeasts have been shown to suppress coliform detection (54,95,117). The significance of coliform suppression in municipal and rural water supplies have been previously presented (1,11,42) and it has been speculated that suppression can at least partly be attributed to elevated SPC numbers. For example, increased SPC densities have been noted prior to, or have been associated with, water-borne disease outbreaks (1,42,73,83). Pathogen occurrences have also been noted to occur in the absence of detectable coliforms in samples with high SPC numbers (1). Data from the National Community Water Supply Study (73) showed that the frequency of detecting coliforms decreased when SPC levels exceeded 500 to 1000 cfu/ml.

The species components of SPC populations as well as cell densities (42) may be important in assessing the propensity for interference with coliform detection (54,62). Identification of the SPC population in one study indicated, on an average, that nearly 3 times more potential coliform antagonists were present in distribution water compared to raw surface water (35% vs 14%) (62). Observations consistent with a possible role of antagonists in coliform suppression were provided by the lack of coliforms in 81% of the drinking water specimens which contained 20% or more antagonists in the SPC population. Conversely, when the antagonist population was less than 20% of the SPC, a greater coliform incidence (57%) was recorded. Hutchinson (54) found that the loss of sensitivity in detecting coliforms as measured by a reduction in MPN values ranged from 28 to 97 percent depending on the combination of strains of SPC organisms. Various hypothesis have been proposed to account for coliform interference including: antibiotic production (117), competition for nutrients (40,42,76,95) and production of toxic waste produces (54,95,116).

The current study was initiated to examine the role of SPC bacteria in the injury process; to elaborate on the mechanism of coliform antagonism and to develop a method for preservation of coliform densities during transport and storage.

Interactions between Coliforms and SPC bacteria

Experiments were first designed to demonstrate coliform antagonism/injury in drinking water. Chlorinated tap water (avg. 1.0 mg/l free chlorine) from the Bozeman distribution system was collected and split into two parts. One part was immediately dechlorinated with sodium thiosulfate, 0.008% final concentration (2). The other part was superchlorinated with an additional 2.0 mg/l free chlorine (prepared from a stock solution of bleach; Serco bleach distributed by S.E. Rykoff Co., Los Angeles, CA) for 1 h at room temperature, then dechlorinated. Superchlorination effectively eliminated all microorganisms from these samples. A culture of *Escherichia coli* was grown 18 h in TLY broth, washed twice and added to the samples at densities of approximately 1 cfu/ml. The superchlorinated sample resulted in essentially a pure culture of *E. coli* while the other sample was a mixture of coliforms and naturally occurring SPC bacteria. Usually, a single species of SPC bacteria became predominant in a water sample. It was possible to identify an interaction between a coliform and a single species of naturally occuring SPC bacteria. Some of the organisms identified in these expepriments included *Pseudomonas* (*P. cepacia*, *P. stutzeri* and *P. maltophilia*) and *Acinetobacter* spp. All SPC enumerations were performed by the spread plate procedure according to *Standard Methods* (2).

Preliminary results of these experiments indicated that depending on the SPC organism, coliforms either died or became injured. Figure 9 demonstrates the interaction between *Pseudomonas cepacia* and *E. coli*. *P. cepacia* had a growth rate of approximately 1 log increase per day. As SPC densities approached 10^4-10^5 cfu per ml, coliform counts decreased at a rate of 0.5 log per day (Figure 9). Coliforms in pure culture (superchlorinated sample) remained constant over the 8 day period. No significant injury was detected in either the mixed or pure culture coliform populations (as determined by TLY and TLY-D media). Statistical analysis of ten replica experiments demonstrated a significant correlation ($r = 0.66$; $p < 0.05$) between the initial SPC level and the rate of coliform decline.

The interaction between *Pseudomonas maltophilia* and *E. coli* produced little decrease in viable counts (over control levels), but 55% injury over a 7 day period (Figure 10). The decline in viable count for the control and mixed coliform cultures was identical but injury was only observed in the mixed culture. Injury did not occur until *P. maltophilia* levels reached 10^4-10^5 cfu/ml.

The ratio of coliforms to SPC bacteria ($1{:}10^4$-10^5) in the above experiments where antagonistic or injurious interactions occurred are similar to the ratios for coliforms and SPC bacteria in drinking water (coliforms 1-10 cfu/100 ml; SPC $>$ 500 cfu/ml) where coliform suppression has been reported (21,40,42).

Attempts to detect bacteriocins or antibiotic production by overlaying irradiated SPC colonies with cultures of four different coliforms (*E. coli*, *Klebsiella pneumoniae*, *Citrobacter freundii* and *Enterobacter agglomerans*) were unsuccessful. While bacteriophage could not be ruled out, a nutritional interaction between SPC and coliform bacteria was thought to be more likely.

Nutrient competition between coliforms and SPC bacteria.

Consistent with the idea of nutritional interaction is the concept of competition between SPC bacteria and coliform organisms for some limiting essential nutrient (54,62,105). Seligmann and Reitler (105) have postulated that as *Pseudomonas aeruginosa* grows and lowers available nutrient levels, *E. coli* becomes more susceptible to bacteriocins. The ratio in which compounds of carbon, nitrogen and phosphorus are required by most heterotrophic bacteria approximates 100:10:1 (C:N:P). Since assimilable organic compounds often limit the growth of heterotrophs, studies comparing minimal organic carbon requirements of SPC bacteria and coliform organisms were initiated.

A laboratory controlled system for studying the interactions of SPC bacteria with coliforms was developed. The system consis-

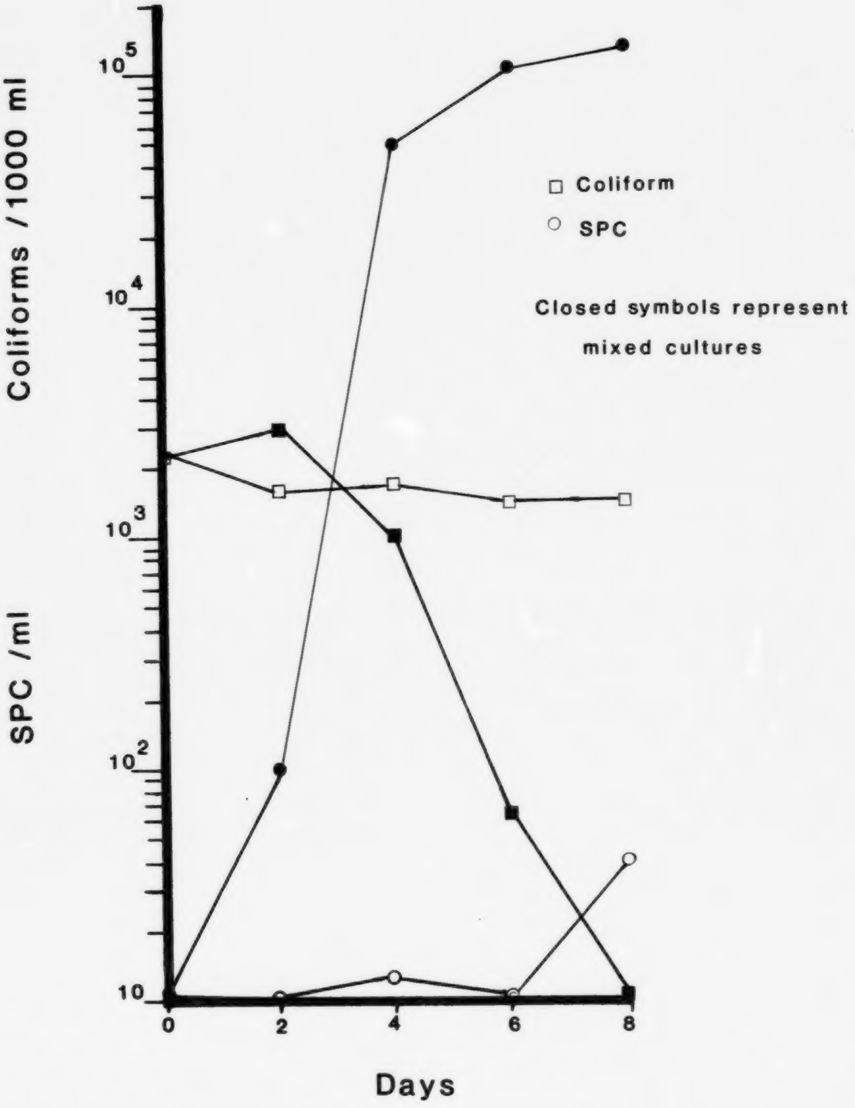

Figure 9. Interaction between Pseudomonas cepacia and E. coli

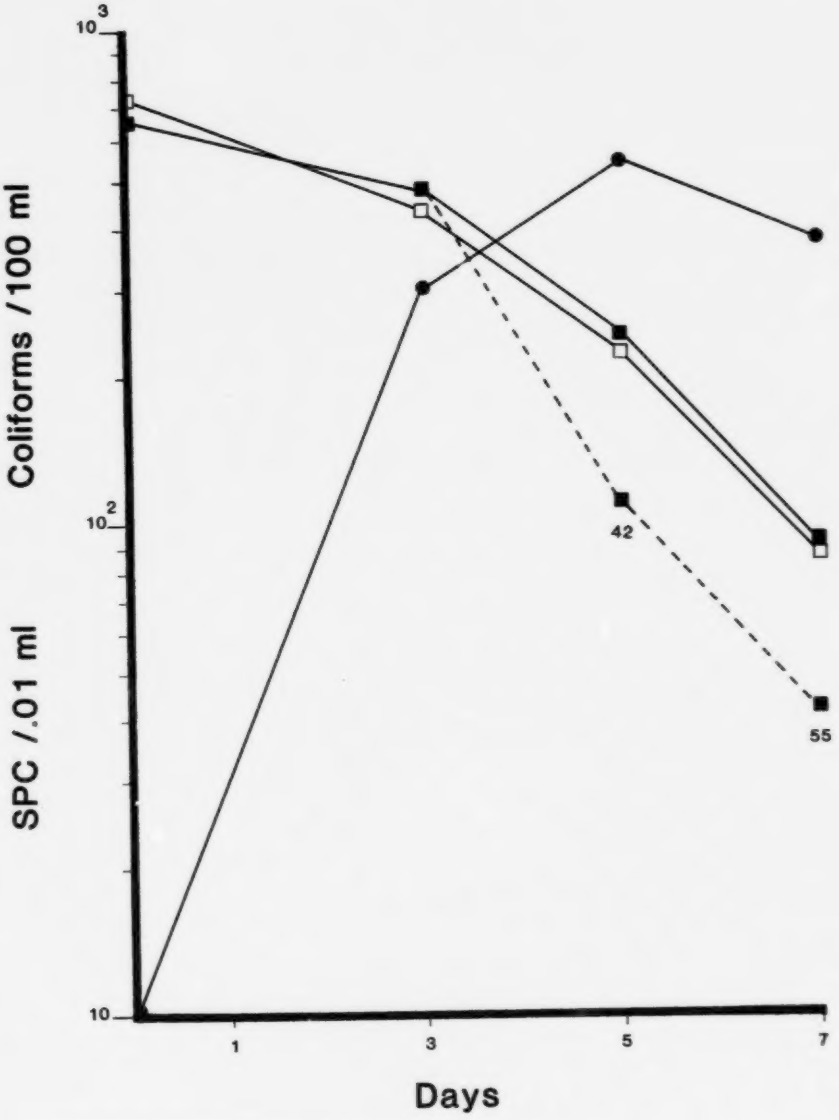

Figure 10. Interaction between *Pseudomonas maltophilia* and *E. coli*. (—□—) *E. coli* control, (—■—) *E. coli* in mixed culture with *P. maltophilia*, (--■--) injured *E. coli* population, (—●—) *P. maltophilia* levels in mixed culture. Numbers represent percent injury to *E. coli* population. No injury was observed in the control culture.

ted of reagent-grade water amended with 0-10 mg/l yeast extract (Difco Laboratories, Detroit, MI). A similar system was set-up with a defined mineral salts medium (Castenholz mineral salts medium, modified so that glucose was the only carbon source) and varying levels of glucose.

Results indicated that SPC bacteria grew at low nutrient levels (0-2 mg/l yeast extract), while coliforms required 4-6 mg/l yeast extract to grow (Table 10). Since coliforms have higher growth requirements than SPC organisms, they are particularly susceptible to nutrient competition. Similar results were obtained with experiments designed to determine the limiting glucose concentration necessary for the growth of coliforms and SPC bacteria. Varying levels of glucose (0-5 mg/l) were added to a modified Castenholz mineral salts medium. The data indicated that glucose concentrations less than 1.0 mg/l limited the growth of *Escherichia coli* cultures (Figure 11). Similar results were observed for cultures of *Klebsiella pneumoniae* and *Citrobacter freundii*. However, a strain of *Pseudomonas stutzeri* isolated from water grew 2.5 logs per day even when no glucose was added (Figure 11). These experiments indicate that *P. stutzeri* is well adapted to an oligotrophic aquatic environment and able to grow rapidly at very low substrate concentrations.

Preservation of Coliform Levels During Transport.

The above studies are valuable in understanding the microbial interactions occurring when water samples are stored or transported. A number of reports in the literature indicate unpredictable changes in coliform levels in stored drinking water (84,85). Nash and Geldriech (85) state that the effect of storage (on coliform recovery) appears to be influenced by the sample temperature during storage, initial coliform density, increase in SPC density, and specific non-coliform bacterial population established in a distribution system (i.e., factors which are marginally controllable and unpredictable). The goal of these experiments was to preserve a water sample so there were no changes in viability and to minimize the amount of injury over a 5 day period (simulating storage/transport conditions).

In order to prevent cells from dying and to allow injured cells to repair themselves, a combination of 0.1% peptone and 0.1% yeast extract was added to water samples. This combination was the most effective in repairing injured cells (data not shown). A number of compounds were tested for their ability to preserve *E. coli* densities during storage (Table 11). Sulfanilamide and p-aminobenzoic acid both failed to inhibit growth even at high concentrations (4.8 and 7.5 g/l respectively). Phenylethanol, rifampicin and nalidixic acid all had narrow ranges where the chemicals were effective in preserving viability and preventing growth. As a result, these compounds were considered unsatisfactory. Chloramphenicol and sodium benzoate were the two most promising inhibitors. At a concentration of 32 ug/ml, chloramphenicol preserved 96.7% viability and only 50.7% injury

Table 10. Minimum Nutrient Levels Necessary for Growth of SPC Bacteria and Coliforms

Organism Tested	mg/l Yeast Extract		
	0-2	4-6	8-10
Escherichia coli	-	-	+
Klebsiella pneumoniae	-	w	+
Citrobacter freundii	-	w	+
Pseudomonas cepacia	+	+	+
Pseudomonas maltophilia	+	+	+

Samples were examined over a three day period for growth above inoculated levels. Symbols: (-) no growth, (w) some small amount of growth, (+) rapid growth.

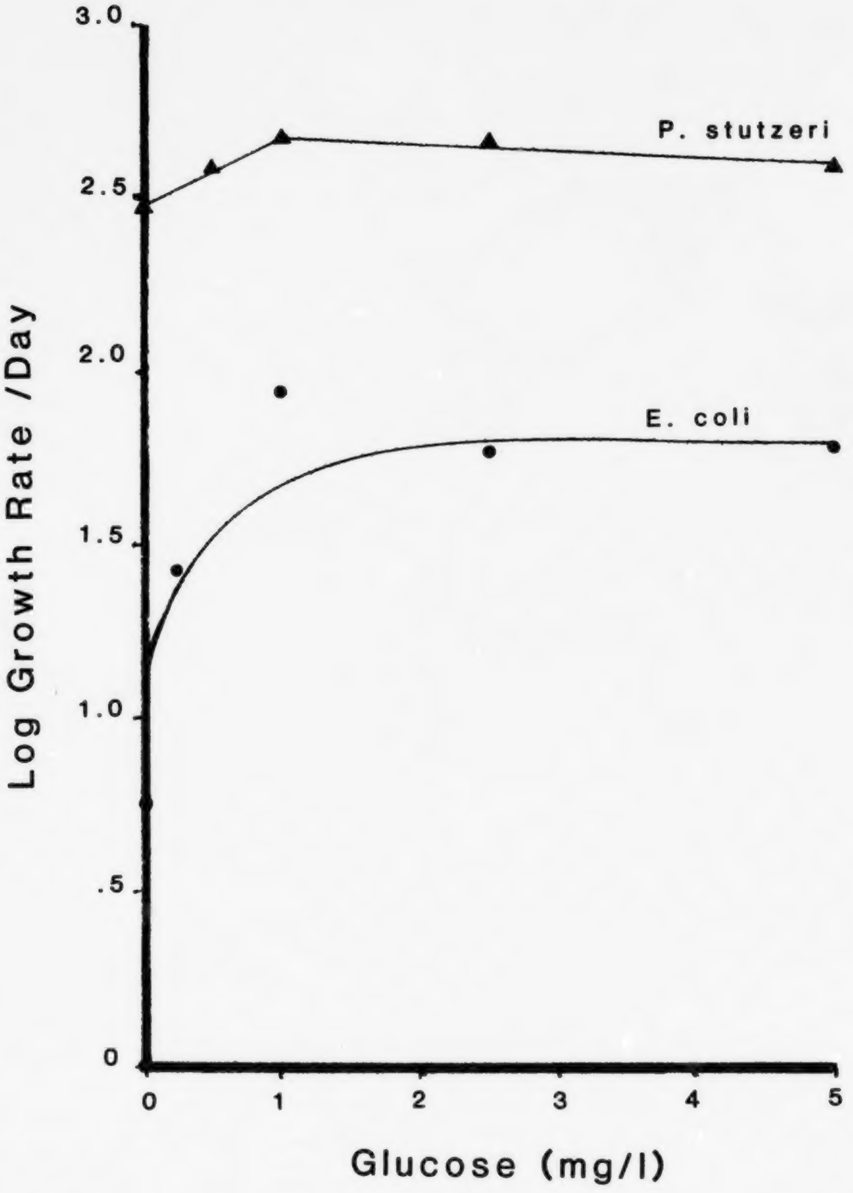

Figure 11. Comparison of growth rates between *Pseudomonas stutzeri* and *E. coli* at low glucose concentrations.

Table II. Inhibitors Used to Preserve E. coli Densities During Storage.

Compound/Concentration		Length of experiment (Days)	% Viability	% Injury
Sulfanilamide	2.5 g/l	1	> 500[a]	-
	5.0 g/l	1	> 500	-
	7.5 g/l	3	130	5
p-aminobenzoic acid	2.4 g/l	1	> 500	-
	4.8 g/l	1	> 500	-
Phenylethanol	2.5 g/l	1	78	14
	5.0 g/l	1	34	95
	7.5 g/l	1	0	-
	10.0 g/l	1	0	-
Rifampicin	0.8 µg/ml	1	> 500	-
	8 µg/ml	1	16	83
Nalidixic acid	0.08 µg/ml	1	> 500	-
	0.8 µg/ml	3	142	58
	0.16 µg/ml	1	0	-
Chloramphenicol	32 µg/ml	6	97	51
	24 µg/ml	6	87	20
	19 µg/ml	6	71	45
	10 µg/ml	6	22	66
Sodium Benzoate	10 g/l			
	(10^6 cells/ml)	5	92	15
	(10^3 cells/ml)	5	56	68

[a] A % viability > 500 indicates cells grew rapidly even in the presence of the inhibitor. This value is similar to the control, unamended cultures.

after an incubation time of six days at room temperature. Sodium benzoate, at a concentration of 10 g/l, preserved coliform viability at 93% over a 5 day period at 22°C while only 15% of the cells became injured. However, when lower cell densities were used (10^3 cells/ml) the viability decreased and the level of injury increased. Cyclohexamide was used to inhibit fungal cells in drinking water (data not shown). While a level of 1-10 mg/l was effective in preventing fungal growth, extensive mutiplication of these organisms is not common in drinking water.

Studies with laboratory grown E. coli indicated that a solution of sodium benzoate (10 g/l), chloramphenicol (32 ug/ml), EDTA (25 mg/l), yeast extract (1 g/l), and peptone (1 g/l) was effective in preserving E. coli densities (80-100% viability) over a 5 day period (Figure 12). Only a minimal amount of injury occurred with this solution. However, when this technique was used to preserve a contaminated surface water sample, extensive background growth occurred. Evaluation of other compounds including sulfanilamide, p-aminobenzoic acid, phenylethanol, rifampicin, NaCl and nalidixic acid did not prevent background growth.

While the results presented here are preliminary, further studies are required to investigate new approaches to eliminate background growth. Such approaches might include metabolic blocks or antimicrobials directed specifically against background organisms. Additional research is required to understand the interactions between SPC bacteria and coliform organisms. Understanding the interactions between SPC bacteria and coliforms as well as being able to inhibit or reduce these interactions would make microbiological analysis of transported water samples more accurate.

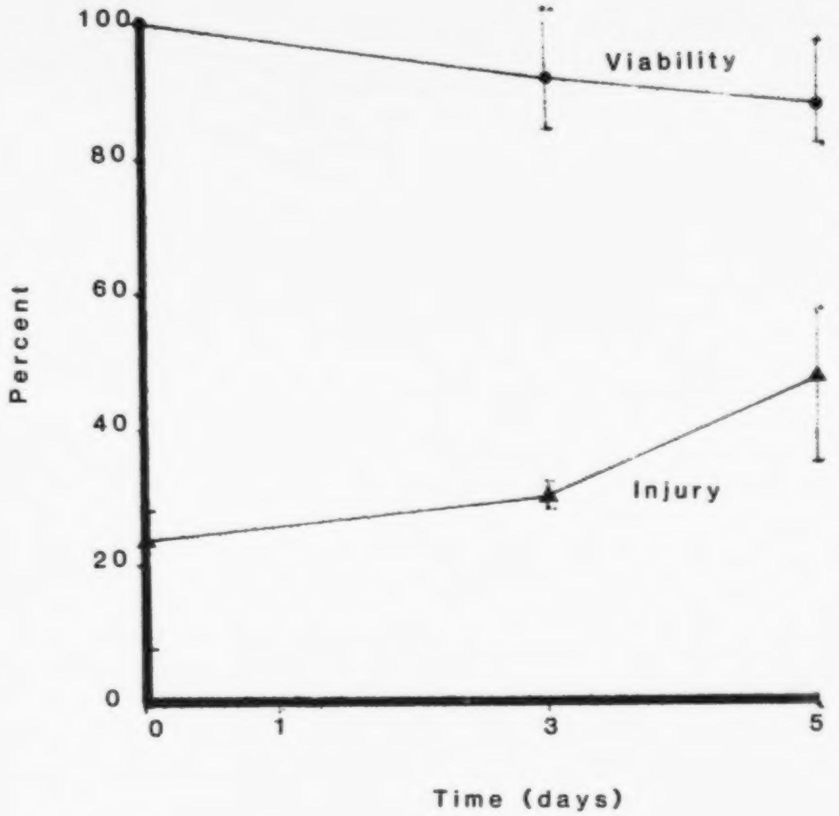

Figure 12. Viability and injury of E. coli in preservation solution.

SECTION 6

EVIDENCE FOR THE ROLE OF COPPER IN THE INJURY PROCESS OF COLIFORM BACTERIA IN DRINKING WATER

SUMMARY

Low levels of copper in chlorine-free distribution water caused injury of coliform populations. Monitoring of 44 drinking water samples indicated 64% of the coliform population were injured. Physical and chemical parameters were measured including 3 heavy metals (Cu, Cd and Pb). Of importance, were copper concentrations ranging from 0.007 to .54 mg/l. Statistical analyses of these factors were used to develop a model to predict coliform injury. The model predicted almost 90% injury with a copper concentration near the mean observed value (.158 mg/l) in distribution waters. Laboratory studies using copper concentrations of 0.025 and 0.050 mg/l in an inorganic carbon buffer under controlled conditions of temperature and pH caused over 90% injury in 6 and 2 days, respectively. Studies of the metabolism of injured E. coli indicate the respiratory chain to be at least one site of damage in injured cells.

INTRODUCTION

The evaluation of potable water supplies for total coliform bacteria is important in determining the sanitary quality of drinking water. Elevated levels of coliform bacteria indicate a contaminated source, inadequate treatment or post-treatment deficiencies. However, the occurrence of injured coliforms presents a significant problem in accurately estimating the number of indicator organisms in drinking water. Published reports have documented that indictor bacteria become injured in drinking water (64), surface water (7), and waste water (12,67). Such debilitated bacteria are not enumerated when currently accepted media are used. In a previous study, we reported the development of a new medium (m-T7) for the improved recovery of injured coliforms from drinking water (64). We found that m-T7 agar recovered 64% more coliforms from potable water supplies than m-Endo agar. These findings document the significance of the media employed in the microbiological surveillance of drinking water.

Coliforms may become injured by exposure to a variety of stressful factors in aquatic environments including: chlorine (17,50), heat (6), freezing (6), acid mine drainage (25), sunlight (37), and heavy metals (6,10,38). Injury of coliform bacteria by chlorine has been well described and is likely the major cause of damaged indicator organisms in drinking water. The usual mechanisms for chlorination, however, can be ineffective in maintaining a free chlorine residual in quiescent portions of some municipal distribution systems and is not regularly practiced in others. Metals represent another potentially important cause of bacterial damage because of their common occurrence in drinking waters (96,111). Little is known concerning the effects of metals on indicator organisms at concentrations likely to be found in drinking water although the effects of heavy metals in sewage and industrial effluents have been investigated where concentrations were extremely high (6,10,38). This study was initiated to determine the role of metals in the injury of *E. coli* under conditions representative of municipal drinking water distribution systems. Our findings support the conclusion that copper may contribute to the injury process and cause significantly reduced enumeration efficiencies of coliform bacteria.

It was also our intention to study the physiological processes involved when coliforms are injured in aquatic environments containing metals. Physiological alterations of *E. coli* stressed by exposure to chlorine have been reported (17). However, there is a paucity of physiological information describing coliforms that have been stressed in the aquatic environment when chlorine is either not present or the concentration is negligible. Respirometry experiments were performed on stressed cells following exposure to metals to investigate possible differences in metabolism between injured and non-injured *E. coli*. These experiments revealed that injured cells have impaired respiratory activity. (A preliminary presentation of these results was presented at the 82nd Annual Meeting of the ASM in March, 1983 [Abstracts of the 82nd Annual Meeting of the ASM, 1983, Q6, p. 261].

MATERIALS AND METHODS

Organism. The *E. coli* strain used in some of the studies was isolated by membrane filtration from the East Gallatin River near Bozeman, MT. The isolate was identified and maintained as previously described (122).

Organism preparation and laboratory injury. Cultures were grown and prepared as before (78). Injured populations were obtained by placing washed cells into a membrane diffusion chamber (75) immersed in water drawn from a lightly used portion of the Bozeman, MT., drinking water distribution system (75). Injury was assessed by differential colony counts on the nonselective TLY agar and selective TLY agar with 0.1% sodium deoxycholate (TLY-D) (78).

Physicochemical analysis of water. Water samples collected in sterile bottles were placed on ice, transported to the laboratory within 1 hr and analyzed within 5 hrs after collection. A complete description of sampling locations and coliform enumeration methods has been previously presented (64). Chemical and physical analysis were performed by accepted methods.

Copper analysis. Samples were acidified with 0.1% nitric acid (final concentration) and sodium nitrate was added as an electrolyte to a final concentration of 0.1 M. The average of 3 replicates was used to determine copper concentrations by the differential pulse polarographic method using a model 374 Polarographic Analyzer (Princeton Applied Research, Princeton, NJ).

Cadmium and lead analysis. The concentrations of these metals were analyzed with a Woodriff Furnace Atomic Adsorption Spectrometer. The average of 4 peak area measurements from replicate aliquots were integrated to calculate each concentration.

Statistical analyses. Statistical analyses including Pearson correlation coefficients and multivariable linear regression models were performed on a Honeywell computer equipped with an SPSS program. Regression models were developed according to accepted techniques.

Oxygen uptake analyses. Oxygraph oxygen uptake mesurements were performed using a Gilson model KM oxygraph equipped with a YSI model 4004 Clark type electrode. The chamber of the instrument was maintained at 30°C. A cell suspension (1.8 ml of 5.0×10^8 CFU/ml) in drinking water was added to the chamber and allowed to equilibrate for 2 min followed by 0.2 ml of phosphate buffered (pH 7.0) substrate [glucose or lactose; 1% final concentration]. The oxygen uptake rates were determined graphically from the slope of the lines as compared with controls without cells (17).

Manometric methods. Additional experiments measuring oxygen uptake were performed with a Gilson Differential Respirometer model IGP-14 as previously described (17) except the bath temperature was maintained at 30°C.

RESULTS

Injury in relation to water chemistry. Preliminary experiments exposing *E. coli* to distribution water with low or negligible chlorine concentration demonstrated greater than 90% injury over a 5 day period. This level increased to 99% with 7 days of exposure. At the same time bacterial viability (CFU's on the non-selective TLY medium) within the test suspension remained high (> 90%) over the entire 7 day period. These initial observations, which agreed with our earlier work (64), prompted further studies to determine the cause(s) of the bacterial injury seen in distribution water where levels of chlorine were either low or non-existant.

A survey was initiated with 44 drinking water samples collected from two drinking water systems where chlorine residuals were habitually low in order to look for relationships between injury and various physicochemical and microbiological parameters. The data obtained are presented in Table 12. In general, the physical and chemical parameters reflect the ground water nature of some of the source waters. Of particular interest are the low free and total chlorine residuals (mean 0.15 and 0.18 mg/l respectively), and the high copper levels (median 0.117 mg/l). Data from this survey were analyzed in an attempt to test

Table 12. Physicochemical and Microbiological Properties of Drinking Water Samples Taken From Several Localities in Southwestern Montana

Microbiological, Physical or Chemical parameter	# of samples	unit of measure	Mean	Minimum	Maximum
Standard plate count bacteria	41	CFU's/ml	110.8	1.0	1270.0
Temperature	44	°C	10.5	4.0	26.0
Turbidity	44	nephlometer turbidity units	0.20	0.01	4.5
Conductivity	44	μ mho	403.1	32.0	650.0
pH	44	pH units	7.7	7.2	8.4
Free chlorine	44	mg/L	0.15	0.00	1.50
Total chlorine	44	mg/L	0.18	0.00	1.60
Phosphate	44	mg/L	.257	0.00	2.20
Nitrite	44	mg/L	.002	0.00	0.10
Nitrate	44	mg/L	1.21	0.05	1.76
Alkalinity[a]	43	mg/L	208.9	171.0	231.0
Hardness[a]	43	mg/L	222.7	88.0	278.0
Total organic carbon	37	mg/l	18.4	0.0	66.8
Copper	36	mg/L	0.117	0.007	0.540
Lead	40	mg/L	0.008	0.001	0.088
Cadmium	40	mg/L	0.001	0.000	0.011

[a] Measured as Ca CO_3 in mg/L.

for correlations between the parameters measured and injury observed in waterborne bacteria. Significant ($p \leq 0.05$) correlations were found for bacterial injury in drinking water and temperature, pH, copper, total organic carbon (TOC) as well as the sum of all metals tested (Table 13). None of the other parameters listed in Table 12 showed a significant simple correlation to injury in drinking water.

Injury is probably a result of complex interactions of various physiochemical processes in drinking water and simple correlations give some, although limited, insight into these complex problems. A computer model was developed, using stepwise multiple linear regression so a more comprehensive approach to bacterial injury in drinking water could be made. All of the parameters listed in Table 12 were used in building the model. Injury, however, was found to be influenced primarily by copper, temperature, pH and alkalinity (Table 14). Among these four factors, copper was the most significant variable while the other parameters probably influence the availability and toxicity of copper ions. The coefficient of multiple determination, R^2, expressed as a percent, indicated that 50.8% of the variation in injury was explained by the variables in the model. Other factors were also shown by computer analysis to affect coliform injury. These factors include TOC, species of coliform as well as free and total chlorine, but these parameters either rarely occurred or were infrequently measured. Therefore, they could not be developed into an adequate model. Other factors that could not be measured, such as duration of aquatic exposure and the physiological state of the organism as it entered the aquatic environment are also thought to be important.

The survey results were verified by laboratory experiments conducted to test the capacity of copper, lead and cadmium to cause measurable injury in a water-isolated strain of E. coli. Based on preliminary water chemistry analyses, metal levels for our first experiments were: copper, 0.25 mg/l; cadmium, 0.017 mg/l; and lead, 0.07 mg/l. Initial experiments showed coliform injury occurred at these metal levels in reagent grade water, a phosphate buffered system (pH 7.0) and in an inorganic carbon buffered system (pH 7.0). The inorganic carbon buffered system was found to be preferred since this buffer is the dominant buffering system of most distribution waters and metals were able to react without interference from phosphate or magnesium. The potential of these metals to cause injury was tested individually, and it was determined that copper alone caused as much injury by itself as a mixture of all the metals examined. The progressive injury of E. coli caused by exposure to copper (.05 mg/l and .025 mg/l) in inorganic carbon buffer is shown in Figure 13. Greater than 90% injury occurred in this system within 2 days (4°C) at 0.05 mg/l copper. After 5 days exposure to 0.05 mg/l copper, cells demonstrated greater than 99.5% injury. With longer contact times at this copper concentration, injured cells started to die, resulting in the slight decrease in injury seen on day 7. At lower copper concentrations (0.025 mg/l), injury

Table 13. Significant[a] Correlation Coefficients for Injury in Drinking Water.

		Injury[b]	
Parameter	Correlation coefficient (r)	No. of samples	Significance (p)
Temperature	0.257	44	.044
pH	0.479	44	<.001
Copper	0.312	36	.032
Total organic carbon	0.301	37	.035
Metals[c]	0.537	44	<.001

[a] Only values significant at the 95 percent level ($p \leq 0.05$) are presented.

[b] Injury is calculated as the difference between verified coliform counts on m-T7 agar minus the verified coliform count on m-Endo agar.

[c] The metals parameter represents the sum of copper, lead and cadmium values observed.

Table 14. Computer Model Derived to Predict Coliform Injury

Model		Coefficient of Multiple Determination (R^2)	Number of Observations (N)	Total Squared Error (C)	F Values
Injury[a] =	-261.7	0.508	30	5.7	-
	+150.0 (Copper)				4.82
	+ 2.04 (Temperature)				3.48
	+ 21.69 (pH)				2.02
	+ 0.314 (Alkalinity)				

[a] Injury is calculated as the difference between verified coliform counts on m-T7 minus the verified coliform count on m-Endo agar.

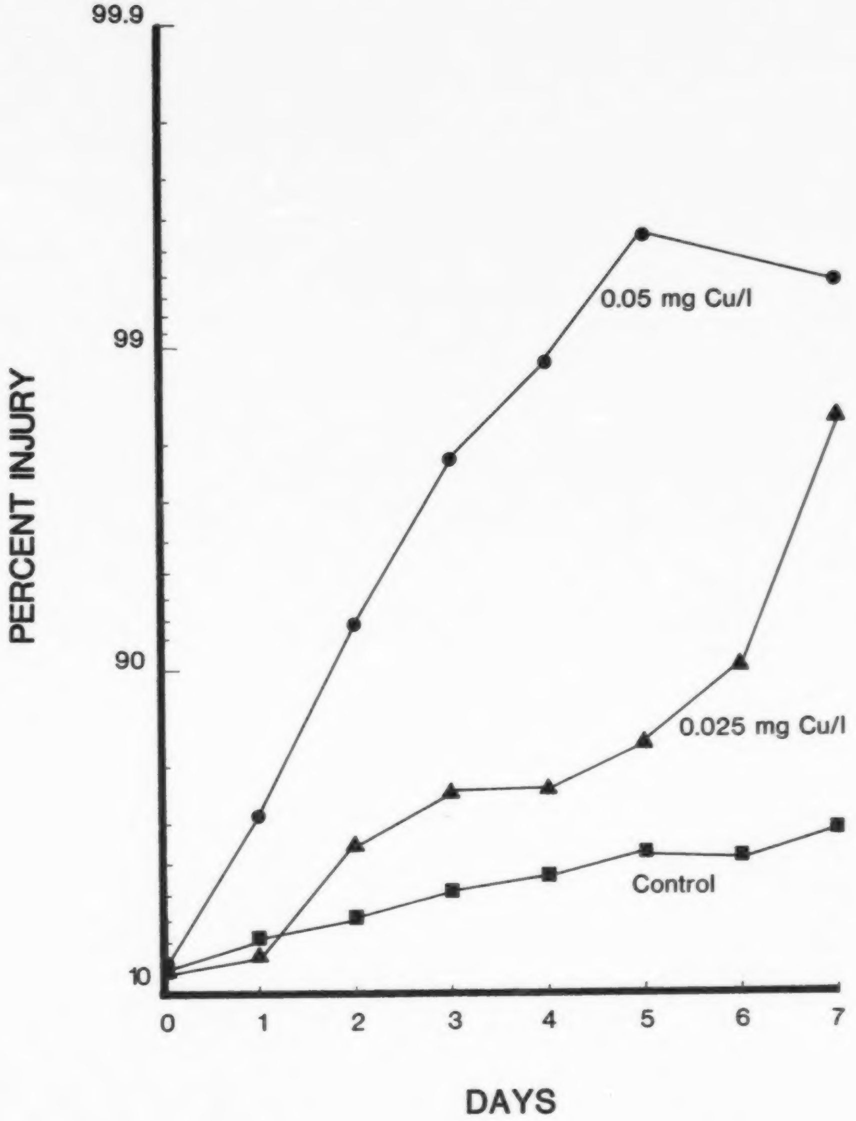

Figure 13. Injury of E. coli exposed to copper in an inorganic carbon buffer. E. coli (1.1×10^5 CFU/ml) were exposed to, (●) .050 mg Cu/L, (▲) 0.025 mg Cu/L and (■) inorganic carbon buffer (1.68×10^{-3} M, pH 7.0) without copper.

progressed slower taking 6 days to reach the 90% level (Figure 13). Increasing temperatures accelerated the rate of injury caused by copper. Although a limited number of experiments were performed, the time necessary to cause 90% injury at 0.05 mg/l copper was only 1 day at 22°C. In addition, higher metal concentrations (0.25 mg/l) caused injury at a faster rate (less than 1 day at 4°C), but large decreases in cell viability were also observed.

Physiology studies. E. coli exposed to distribution water from a quiescent portion (i.e., no chlorine detected) of the Bozeman drinking water distribution network in membrane diffusion chambers were examined to determine the physiological implications of aquatic injury. Initial experiments revealed a trend of decreasing oxygen use with increasing injury. Bacterial oxygen utilization (with lactose) measured on a Gilson oxygraph over a short time period (approximately 5 min) decreased 73-83% when cells were injured 70-99.9% (Figure 14). Oxygen utilization when cells were grown on glucose decreased 50% for injured cells (Figure 14). Similar results were obtained with long term (2 h) respirometry experiments (Figure 15). Bacteria incubated in an enriched medium containing either glucose or lactose required approximately 30 min (14% injured cells), 55 min (55% injured cells) or 100 min (95% injured cells) to utilize 10 ul $O_2/10^8$ viable cells. This oxygen utilization level was never achieved by cells that were 99.5% injured regardless if they were grown on glucose or lactose (Figure 15). Similar results were found when a mineral salts medium containing either glucose or lactose was used.

Isolation rates for injured cells grown under aerobic and anaerobic conditions also lend support to the above experiments. Data in Table 15 show that injured E. coli were isolated in approximately equal numbers under both aerobic and anaerobic conditions. These results indicate that the presence of oxygen is not required by injured cells during the recovery process.

DISCUSSION

The results of this report showed that E. coli can become significantly injured within two days by exposure to drinking water in the absence of chlorine. The levels of metals found in drinking water samples in the Bozeman area were not unusually high and were similar to those reported in other localities (111). In a study of selected trace metals in household tapwater samples in Dallas, Texas; copper was reported to average 0.037 mg/l (minimum, 0.004 mg/l; maximum, 0.164 mg/l) (111). A report of metals in finished water supplies of 100 largest cities in the United States found copper levels ranging from < 0.006 to 0.250 mg/l (111). Another report of 380 finished drinking waters in the United States found copper in 65.2% of the samples averaging 0.043 mg/l (111). Data from Figure 13 indicate that coliforms in many of the nations potable water supplies would become injured within 2-3 days because of exposure to metals naturally

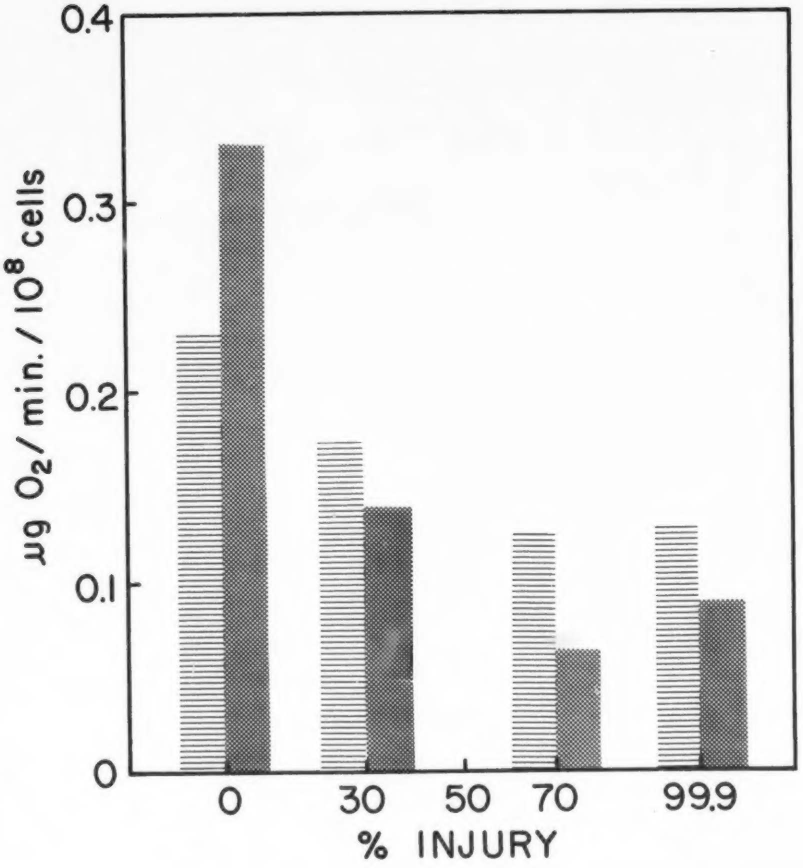

Figure 14. Short term oxygen uptake by injured and healthy *E. coli*. Substrates used were 1.0% glucose (▤) and 1.0% lactose (▩). Oxygen utilization was measured by using a Gilson model KM oxygraph equipped with a YSI model 4004 Clark type electrode.

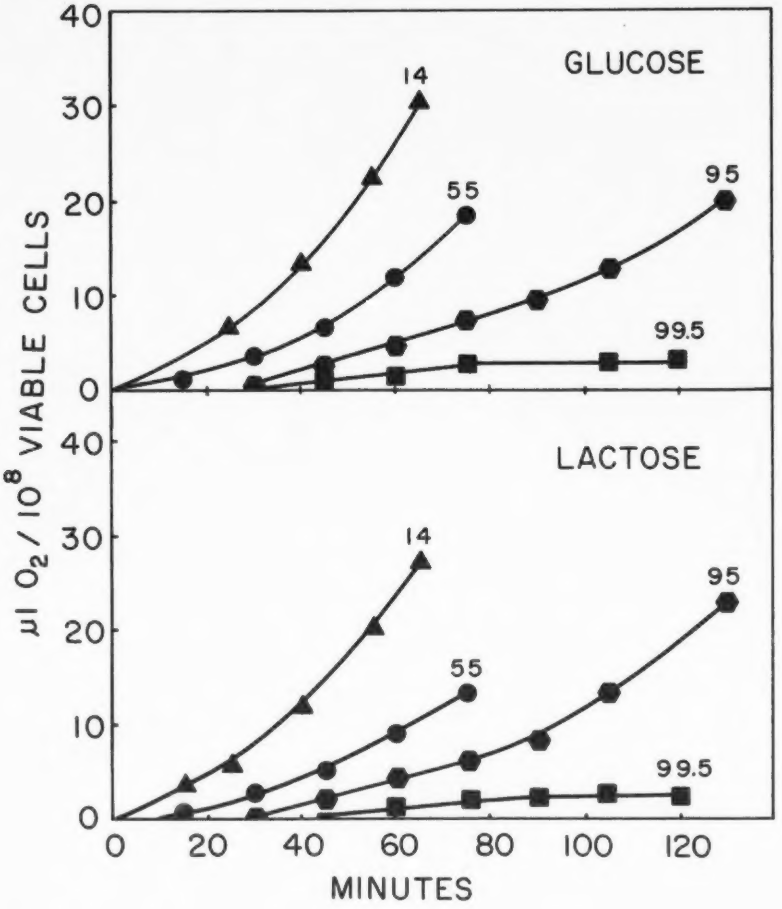

Figure 15. Oxygen uptake by E. coli suspension stressed in water. The medium used was tryptic soy broth plus either 0.5% (wt/vol) glucose or 0.5% (wt/vol) lactose. The numbers following the data curves are percent injury. Oxygen utilization was measured with a Gilson Differential Respirometer model IGP-14.

Table 15. Aerobic-Anaerobic Plate Count Data with Injured *E. coli*.

# Days Stress	AEROBIC			ANAEROBIC		
	TLY[a] Count	TLYD[b] Count	%[c] Injury	TLY[a] Count	TLYD[b] Count	%[c] Injury
0	1.13×10^{9}	9.97×10^{8}	11.8			
2	9.33×10^{8}	3.96×10^{8}	57.5			
4	8.17×10^{8}	1.81×10^{8}	77.9	7.30×10^{8}	2.30×10^{8}	68.2
6	2.10×10^{8}	7.43×10^{6}	96.5	1.80×10^{8}	7.70×10^{6}	96.17
0	1.21×10^{9}	1.02×10^{9}	16.4	1.08×10^{9}	1.01×10^{9}	7.3
2	8.80×10^{8}	6.70×10^{8}	23.9	8.46×10^{8}	5.73×10^{8}	32.3
4	5.10×10^{8}	1.40×10^{8}	72.6	6.80×10^{8}	1.83×10^{8}	73.0
6	1.87×10^{8}	1.51×10^{7}	92.0	2.20×10^{8}	1.17×10^{7}	94.6
7	9.50×10^{7}	3.16×10^{5}	99.7	1.97×10^{8}	5.00×10^{5}	99.8

[a] TLY is Tryptic soy broth w/o dextrose + 0.3% Yeast extract + 1.0% lactose + 1.5% agar

[b] TLYD is TLY + 0.15% desoxycholate

[c] $\%\ \text{injury} = \frac{\text{TLY count} - \text{TLYD count}}{\text{TLY count}} \times 100$

found in drinking water. Sources of metals in distribution waters include copper and brass material in pumps, pipes, valves and other appurtenances (111). Copper may also be introduced as copper sulfate, used to treat algal problems in reservoirs and as a trace contaminant added with flocculation material (111). In addition, copper may enter surface water from geological sources. This process may be especially important in some areas (i.e., Montana) where copper is naturally abundant.

Statistical modeling of 30 drinking water samples indicated the complex interaction of ambient chemical and physical factors (Table 14). Temperature, pH and alkalinity were implicated as parameters that affected the availability of copper. These statistical models can also be used to predict the impact of copper concentrations on coliform injury in drinking water. By holding the parameters of temperature, pH and alkalinity of the mean observed values in drinking water (Table 12) constant, we can evaluate the role of copper alone as a causitive factor for injury. At the lowest copper concentration observed in distribution water (0.007 mg/l) the model predicts no injury. Near the mean copper concentration observed (0.158 mg/l), the computer model predicts a 10 fold (1 log) difference between an injurious (m-Endo) and a noninjurious (m-T7) medium. At the highest copper level measured (0.54 mg/l), the model predicts almost a 100 fold (2 log) underestimation of actual coliform densities due to injury (Table 14).

Data presented in this report have several important implications in the areas of sample collection and coliform methodology. *Standard Methods for the Examination of Water and Wastewater* (2) and the EPA *Handbook for Evaluating Water Bacteriological Laboratories* (38) both specify adding ethylenediaminetetraacetic acid (EDTA) as a chelating agent to sample bottles used to collect water, wastewater or industrial effluents "high" in heavy metals. Similar instructions are given for drinking water samples in the manuals *Handbook for Sampling and Sample Preservation of Water and Wastewater* and, *Microbiological Methods for Monitoring the Environment* (10). These specify the addition of EDTA (372 mg/l) to bottles used to collect samples containing > 0.01 mg/l concentrations of heavy metals (10). Since the results of various studies (111), including this report, indicate that most drinking water contains > 0.01 ug/l concentrations of copper alone, our recommendations are that EDTA be added routinely to all bottles used to collect water samples especially if these samples will be stored or transported. We found that EDTA at a concentration of 25 mg/l was sufficient to prevent injury caused by copper (0.05 mg/l) over a 5 day period (data not shown).

Physiological aspects of this type of chlorine independent aquatic injury were investigated to determine the metabolic lesion. Distribution water injured *E. coli* show a decreased use of oxygen and are likely to depend on fermentation pathways during recovery. This observation leads to the speculation that the cellular damage might be in the respiratory chain. This is a likely target since the respiratory enzymes are membrane-associated. Previous work by Camper and McFeters (17) suggested chlorine also attacked the cell envelope in *E. coli* and caused reduced active transport capabilities as well as affecting the respiratory process. Copper could conceivably bind to thiol groups of respiratory enzymes near the cell surface and inhibit their function.

SECTION 7

CAUSES, IMPLICATIONS AND METHODS FOR ENUMERATION OF INJURED COLIFORMS IN DRINKING WATER

INTRODUCTION

Stress resulting from a variety of chemical and physical environments has been observed in indicator bacteria. The importance of injured bacteria was recognized in the food industry as early as 1935 by Mudge and Smith (81) and in 1954 by Heinmets, et al. (47) when they reported using certain metabolic intermediates to restore the viability of chlorine-inactivated *Escherichia coli*. Workers in the area of water microbiology are also gaining an appreciation for the importance of stressed indicators in the assessment of water quality. The purpose of this report is to review the sources of injury, influencing factors, enumeration methods and the importance of enumerating stressed coliforms with special emphasis on the problems associated with drinking water. While this report is not intended to be comprehensive, our aim is to give a perspective regarding past investigations and insight into the needs for future research relating to injured bacteria in drinking water.

Definition of Injury in Water

Injury has been defined as the sublethal physiological consequence of exposure to stresses which cause a loss in the ability of microorganisms to grow normally under conditions satisfactory for untreated cells (6,7,17,78). Injury has also been described as reversible; that is, under proper conditions of temperature and nutrients, injured organisms can repair the cellular lesion and become indistinguishable from unstressed cells (6,7,17,78). This sublethal damage has been observed as an inability of bacteria to proliferate on media containing selective agents that have no apparent inhibitory action on undamaged cells (6,7,49,78, 94,97,103). As a result, most stresses that are applied sublethally will result in damaged cells incapable of growing on selective media commonly used in aquatic microbiology.

Sources of Injury in Water

Although our understanding of all potential sources of injury in water is incomplete, several areas have been extensively studied.

Chlorine has been the most widely recognized cause of injury to coliform bacteria in drinking water (2,12,17,44,45,67,68,74, 80,81,87,88,114,121). Both the chlorine dose and the time of exposure have been shown to influence the degree of injury in coliform organisms (17,74). Experiments performed in our laboratory examining the reversibility of chlorine-induced injury in *E. coli* under conditions comparable to drinking water, indicate that most coliforms exposed to sodium hypochlorite are rapidly killed once the chlorine demand of the system is satisfied. However, a sizable portion of the survivors are injured in such a way that they fail to grow under selective conditions commonly used to detect these organisms in drinking water (Figure 16). Because of our past lack of adequate techniques to measure injury, the extent of coliform stress in drinking water is largely unknown, although some reports estimate that coliforms are recovered with efficiency of 10% or less in chorinated aquatic environments (7,8,12,17,29,75,76,78).

Physiologically, chlorine causes damage to the bacterial cell membrane (17,45,74,114,122,123). Experiments have shown respiration, glucose transport, and ATP levels all decrease in injured populations (17,45,74,114). Electron microscopy of injured cells has demonstrated morphological changes in the cell membrane (123). Chlorination has also been reported to cause death due to unbalanced metabolism (121), affect protein synthesis and amino acid structure (5,88), as well as cause genetic defects by modifying purine and pyrimidine bases (45,51,55,87,106).

Although little data exists describing injury from other water disinfection agents such as ozone, chlorine dioxide and ultraviolet irradiation, it has been suggested that these treatments also cause injury (6,15,98). Chlorine dioxide has been reported to inhibit membrane bound enzymes and protein synthesis (5,98). Ultraviolet irradiation is known to cause damage to nucleic acids as well as alter membrane functions (6).

Environmental factors such as heat, freezing and sunlight are also known to cause injury (6,15,37,53). Exposure to sunlight can inactivate 90% of the indicator bacteria in stream water within 13 to 32 min (37). Although these parameters are probably of little importance in distribution waters, they may affect coliforms in source waters and reservoirs. pH is another environmental factor that may cause injury to indicator bacteria in surface waters as a result of acid rain or acid mine drainage (6,25) and from lime or soda additions in the treatment plant. pH is known to affect substrate transport across membranes (6) and also alters the availability of heavy metals.

Recently, the role of transition metals in the injury process in drinking water was described (26). A combination of laboratory studies and field data indicated that copper, at concentrations normally found in drinking water, could cause significant injury to coliform bacteria. While other metals such as cadmium, lead and zinc were also detected in drinking water samples, these metals or combinations of these metals were not as

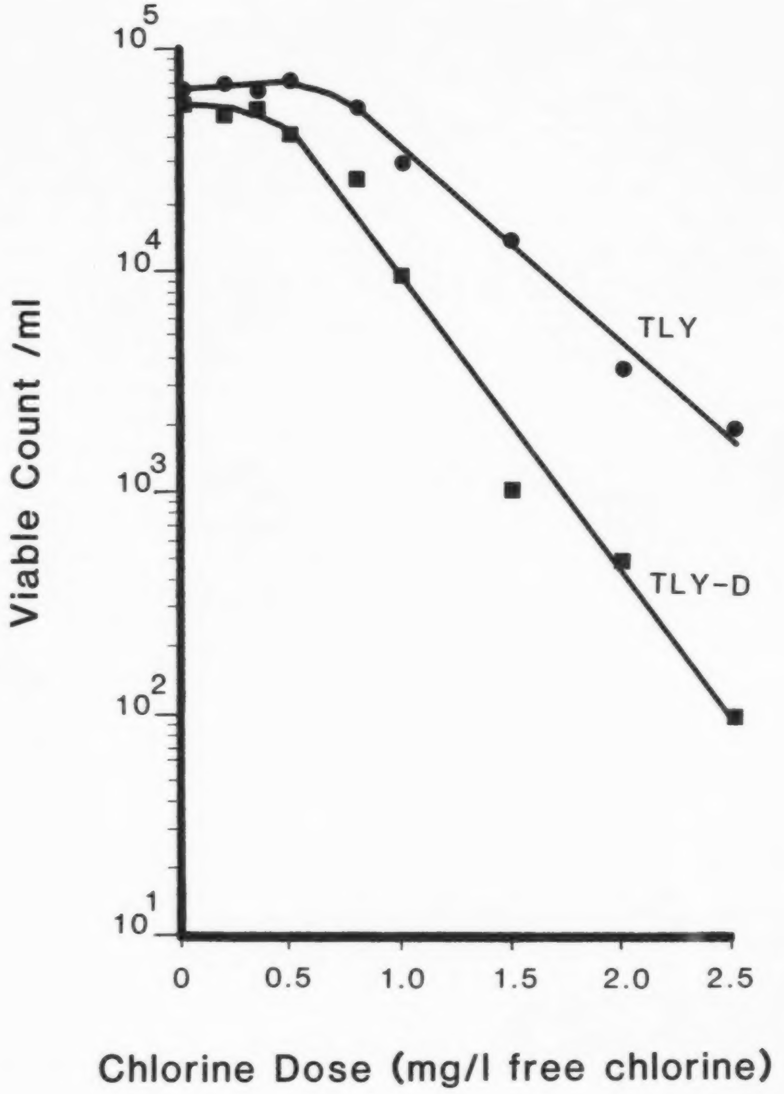

Figure 16. Relationship between chlorine dose and injury to Escherichia coli. Cells were exposed to varying levels of sodium hypochloride for 10 min. The chlorine demand of the unwashed cells in reagent grade water was 0.4 mg/l chlorine. Percent injury is calculated as the difference between TLY counts and TLY-D counts by the equation:

$$\% \text{ injury} = \frac{\text{TLY count} - \text{TLY-D count}}{\text{TLY count}} \times 100$$

important in the injury process as copper alone. Copper has been detected in 65.2% of 380 finished drinking water supplies in the United States with a mean concentration of 0.043 mg/l and a range of 0.001 - 1.060 mg/l (111). Results from experiments performed in our laboratory indicate that 90% injury could occur in coliform populations within 2 days at copper concentrations near the national mean (Figure 17). Statistical analysis of 30 drinking water samples suggested that pH, alkalinity and temperature also influenced the amount of injury, presumably by altering the chemical species and activity of the dissolved copper. Other factors such as total organic carbon levels, and the species of coliform present are thought to influence injury due to copper. Physiologically, copper-injured cells show impaired respiratory activity and are unable to utilize oxygen during the repair process (26). Sources of copper in drinking water include copper pipe material, brass, faucets, and other attachments (111). Copper may also be introduced as copper sulfate, used to treat algal problems in reservoirs and as trace contaminants added with flocculation material. In addition, copper may also enter water from geological sources. This process may be especially important in some areas (i.e., Montana) where copper is naturally abundant.

Another source of injury in drinking water results from biological interactions. Some strains of heterotrophic or "standard plate count" (SPC) bacteria in drinking water have been shown to suppress coliform detection (40,42,54,95,117). The significance of coliform suppression in municipal and rural water supplies has been previously presented (1,11,42) and it has been demonstrated that suppression can at least partly be attributed to elevated SPC numbers. For example, increased SPC densities have been noted prior to, or associated with, waterborne disease outbreaks (1,42,73,83). Pathogen occurrences have also been documented in the absence of detectable coliforms in samples with high SPC numbers (1). Data from the National Community Water Supply Study (73) showed that the frequency of coliform detection decreased when SPC levels exceeded 500 to 1000 cfu/ml.

In addition, interactions of SPC bacteria with coliforms may also cause injury. For example, experiments in our laboratory showed that the interaction between _Pseudomonas maltophilia_ and _E. coli_ produced little decrease in viable counts (over control levels), but produced 55% injury over a 7 day period (Figure 18). The decline in viable bacteria for the control and mixed coliform cultures was identical but injury was only observed in the mixed culture. Injury did not occur until _P. maltophilia_ levels reached 10^4-10^5 cfu/ml. The ratio of coliforms to _P. maltophilia_ (1:10^4-10^5) in the above experiments where injurious interactions occurred are similar to the ratios for coliforms and SPC bacteria in drinking water (coliforms = 1-10 cfu/100 ml; SPC > 500 cfu/ml) where coliform suppression has been reported (21,40,42). Attempts to detect bacteriocins or antibiotic production by overlaying irradiated SPC colonies with cultures of four different coliforms (_E. coli_, _Klebsiella pneumoniae_, _Citrobacter freundii_ and Enterobacter agglomerans) were unsuccessful. While bacteriophage could not be ruled out, a nutritional interaction between SPC and coliform bacteria was thought to be more likely. Such interactions are important particularly when samples are transported and/or stored. Previous studies have shown that coliform levels may vary significantly during sample transport (84,85), but injury in these experiments was not controlled or examined.

Finally, there are many toxic organic substances that may be dissolved in surface waters and their role in the injury process is completely unknown. As our awareness of injured coliforms in drinking water increases, other sources of injury will become more apparent.

Influencing Factors

In addition to the primary causes of injury in drinking water, a number of factors can influence the extent or severity of coliform stress. These factors include environmental parameters as well as sample handling and enumeration methodologies.

Environmental factors that influence the extent of injury include time and temperature of exposure of the indicator bacteria in water. As mentioned previously, over 90% injury can occur in a coliform population within two days when exposed to 0.05 mg/l copper. Similarly, Bissonnette et al. (7) showed that E. coli became over 90% injured when exposed 2 days in certain mountain streams. Other investigators have reported 97% injury in E. coli populations exposed to reagent-grade water for 3 days (122). Results of experiments where 230 membrane diffusion chambers containing bacteria (75) were immersed in Bozeman, Montana, drinking water indicate that 90% injury occured in coliform populations within 2-4 days (data not published). Generally, as the temperature of the water increased, the time necessary to produce significant levels of injury decreased (3,23,97). Temperature was also shown to be an important variable in a computer model developed to predict injury in drinking water (26). These findings might be explained by the observation that higher water temperatures increase the activity of injurious agents such as chlorine, metals, and biological antagonists. Other environmental factors that probably influence the extent of injury include the bacterial strain, physiological state of the organism, and the concentration of dissolved nutrients, although little information regarding these factors is available.

The way a water sample is collected and handled prior to enumeration greatly influences the viability of injured bacteria in that sample. Sodium thiosulfate is usually added to drinking water samples in order to neutralize any chlorine residual (2,10,38). A chelator such as EDTA should also be added to remove heavy metals, particularly if there is an extended period between sample collection and enumeration (10,26,38).

Laboratory manipulations involving exposure to diluents may

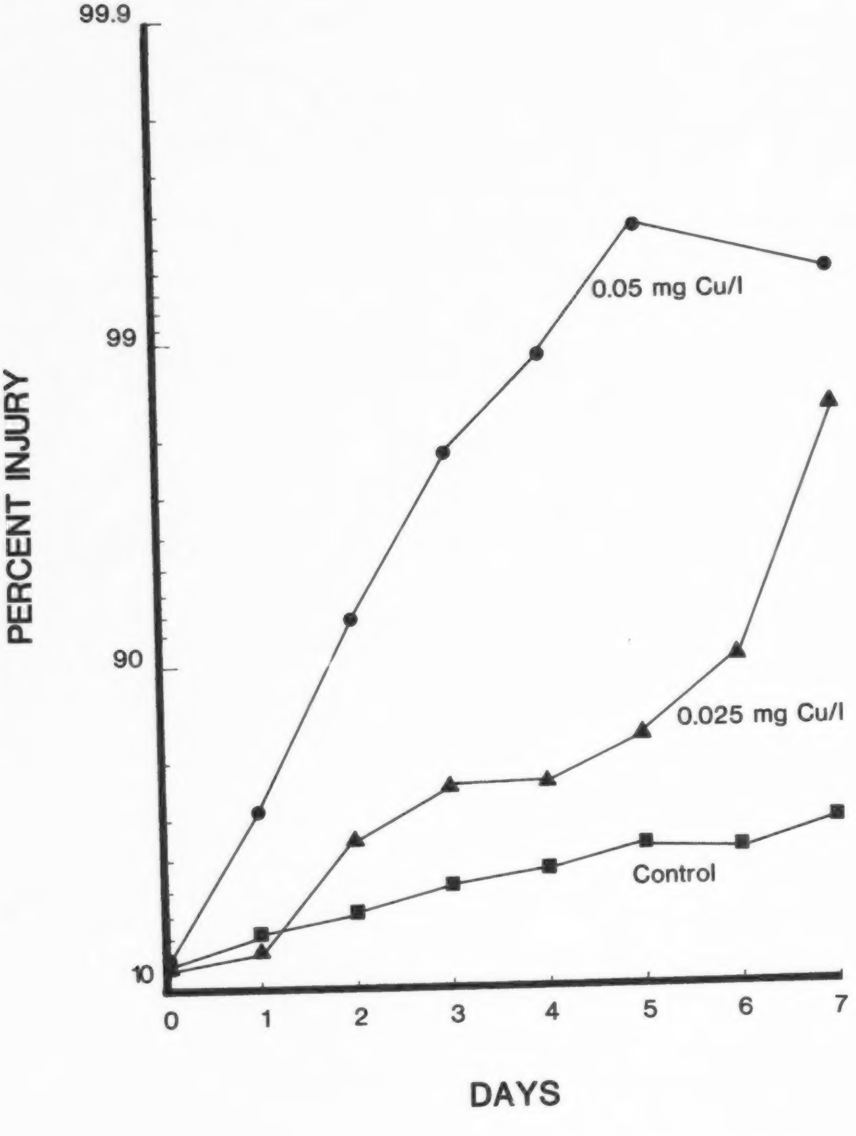

Figure 17. Injury induced by copper at concentrations similar to levels found in drinking water. Cells were injured by placing a washed suspension of E. coli (10^8 cfu/ml) in carbonate buffered reagent grade water (pH 7.0). This suspension was amended with various levels of copper (as $CuSO_4 \cdot 5H_2O$) and incubated at 4°C for time periods up to 7 days. (Adapted from ref. 26).

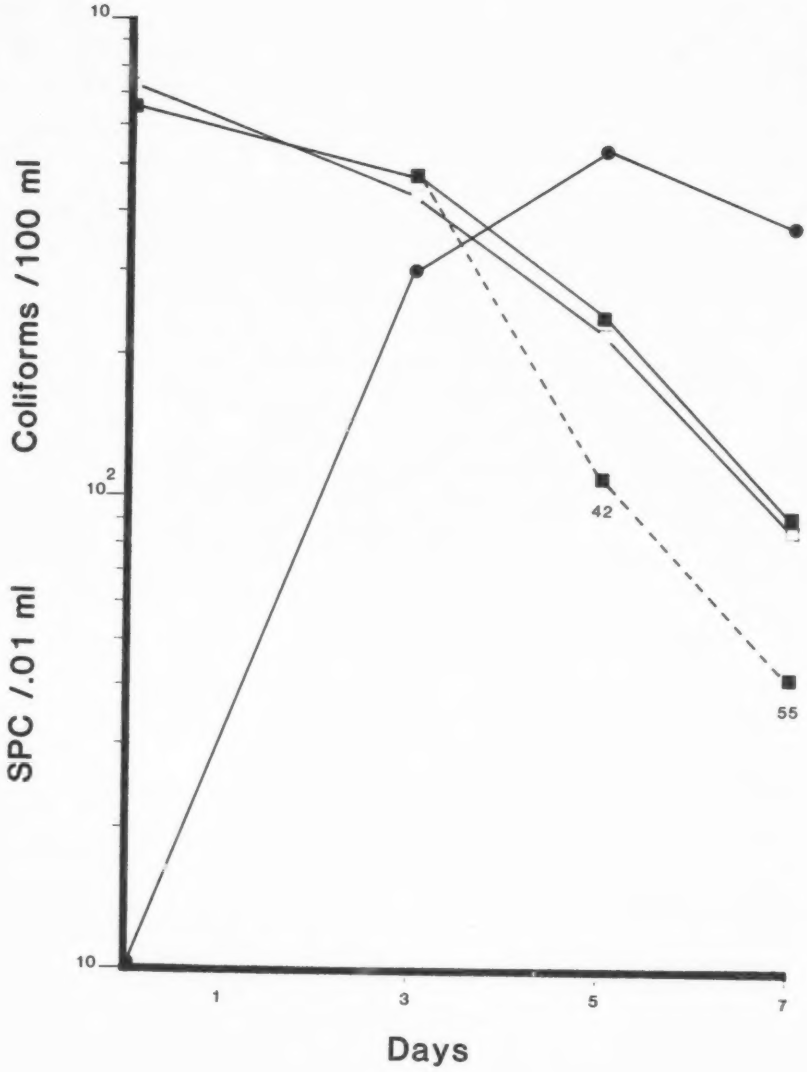

Figure 18. Interaction between Pseudomonas maltophilia and E. coli. (—□—) E. coli control, (—■—) E. coli in mixed culture with P. maltophilia, (•--■---) injured E. coli population, (—●—) P. maltophilia levels in mixed culture. Number represents percent injury in E. coli population. No injury was observed in the control culture.

cause further underestimations of bacterial densities in surface water (78). If diluents are maintained at approximately refrigerator temperatures (c.a., 4°C) their composition and exposure time are of minimal impact on enumeration efficiency. However, substantially lower enumeration recoveries of injured bacteria has been associated with "room temperature" and extended exposure time (78). The enrichment of diluents with low concentrations (0.05-0.1%) of organic material such as peptone, gelatin, tryptone or milk has been demonstrated to be of value in the enumeration of stressed aquatic bacteria (7,12,16,56,59,94,109,118-120). In addition, diluents containing magnesium ($MgSO_4$ or $MgCl_2$) and phosphate (K_2HPO_4 or KH_2PO_4) (2) are known to help recover metabolically injured cells (38).

The choice of laboratory methodology probably has the greatest impact on the enumeration of injured coliform bacteria. Some of the factors to be considered when choosing a method to enumerate injured cells include: membrane filter surface pore morphology, the enumeration technique (MPN or MF), and composition of the selective medium.

Dissatisfaction with the performance of membrane filters was summarized by reports that some types of membrane filters were more effective in the recovery of waterborne fecal coliform bacteria from a variety of sources (9,39,48,90,112). A subsequent study by Sladek et al. (108) identified surface pore morphology as the critical physical characteristic of membrane filters that influenced fecal coliform recovery on selective media in the high temperature (44.5°C) test. Although one report (48) indicated that chlorine-injured total coliforms were recovered less efficiently with membrane filters than by spread plating, other investigators showed that the filter surface morphology was not important in the recovery of indicator bacteria at 35°C (69,78,113). While membrane filter surface pore morphology may not be of great importance for the enumeration of total coliforms in drinking water (78), it may play a role in some instances and certainly will influence the efficiency of the fecal coliform procedure.

When selecting an enumeration technique (MPN or MF) to measure coliform densities in drinking water, a number of factors should be considered. These include the equipment available, time and personnel factors, familiarity and ease of operation. The membrane filter technique has gained wide acceptance because the procedure is simple, rapid, precise, and gives definitive results. However, factors such as turbidity (36,40,50,63), and high numbers of noncoliform bacteria (20,21,40,42,54,62,116,117), may severely reduce the sensitivity of the procedure. In addition, the MPN test is usually regarded as the reference technique, especially if the membrane filter technique has not been previously used (2). The discrepancies between the membrane filter technique and the MPN method first prompted researchers to investigate the problem of injured organisms in aquatic samples (9,40,67,68,107). Since that time, many advances have been made in the membrane filter technique, but little attention has been directed to the effectiveness of the MPN method in the recovery of injured coliforms. A recent study suggested that the presumptive media used in the MPN procedure (lauryl tryptose broth or lactose broth) are inhibitory to injured cells (78). Lactose broth recovered an average of 72% of the injured coliforms (range; 47-98%), while lauryl tryptose broth enumerated stressed indicators at an average rate of 56% (range; 34-79%). Other investigators have confirmed these results and suggested that time released capsules added to MPN tubes would allow for repair of injured cells before selective ingredients reached inhibitory levels (46,61). Other problems with the MPN technique are summarized in reports by Evans et al. (34) and Seidler et al. (104).

Interest in membrane filter techniques for the bacteriological examination of water became widespread in the United States and Great Britain following a number of studies in the 1950's (39). Since that time, much research has been done regarding the membrane filter technique, and many publications and reviews are available (9,13,30). The membrane filter technique is highly reproducible, can be used to test relatively large volumes of water, and yields definite results more rapidly than the MPN procedure (2).

Several recent studies (31,32,49,72) have shown that various selective media used with the membrane filter method yield markedly different coliform enumeration results. One report (78), found that media widely used for water quality estimations (m-Endo, M-FC, EMB and MacConkey agar) generally ranked in the lower half of all media tested with recoveries of injured coliforms ranging from 30 to 70%. Injured coliforms have been shown to be more sensitive to selective (surface-active) ingredients in various enumeration media (7,8,43,49,71,103), presumably because of damage to the bacterial envelope (122,123). In particular, control (uninjured) cells are virtually unaffected by concentrations of desoxycholate or bile salts of 0.1% or less while injured cells are severely inhibited by greater than 0.05% desoxycholate (78). It is interesting to note that all of the media used to enumerate fecal coliforms, and most of those used for total coliforms contain desoxycholate, bile salts, oxgall or lauryl sulfate (27,28,44,68,91,99,110). Dissatisfaction with current m-Endo formulations was summarized in a recent report (64). In addition to recovering as few as 30% of the injured coliforms in drinking water, a number of other problems were also reviewed. These included an inability to distinguish coliforms from non-coliforms on the basis of sheen production (35,52,102), low verification of typical green (golden) sheen colonies (29,35,41) and problems with future availability and quality of basic fuchsin (Geldreich, personal communication).

Temperature of incubation is a selective measure used for the isolation of fecal coliforms from drinking water and wastewater. Researchers over the past decade have recognized that a shift from ambient water temperature to 44.5°C produces dramatic decreases in fecal coliform recoveries. As a result, various resuscitation techniques, including preincubation at 35-37°C for several hours on a non-selective medium, have been proposed (44,68,91,99,110). Resuscitation procedures for total coliforms are also available. Standard Methods (2) specifies an enrichment technique where an absorbent pad is saturated with 1.8 to 2.0 ml lauryl tryptose broth and filters are incubated on this pad 1.5 to 2 hr before transfer to m-Endo agar. While this technique has resulted in some improved recoveries (2,9), other researchers have shown little benefit (35,43). One study showed that lauryl tryptose broth was actually more inhibitory to injured coliforms than m-Endo agar (78). In addition, many microbiologists have found the enrichment technique time consuming and inconvenient. Clearly a need exists for a new medium, specifically designed to improve the recovery of injured coliforms in drinking water.

New Methods for Enumeration of Stressed Coliforms

Recently, new membrane filter techniques have been proposed for the enumeration of stressed total and fecal coliforms (64,65). Steps taken in the formation of a new selective agar medium (m-T7) have ensured that it is selective while minimizing the inhibition of stressed coliforms. The formulation and preparation instructions for m-T7 agar are shown in Table 16. The effectiveness of m-T7 agar was demonstrated by recovering an average of 43% more verified coliforms from 67 surface and drinking water samples than the standard m-Endo membrane filter technique (64). Overall, m-T7 agar recovered significantly more ($p < 0.05$) coliforms than either the standard m-Endo or m-Endo with LTB resuscitation techniques. In one drinking water sample, m-T7 agar enumerated 17 confirmed coliforms while the m-Endo + LTB method recovered none. From the 44 drinking water samples analyzed, m-T7 agar detected 2.7 times more coliforms (a 63% increase) than the m-Endo resuscitation technique and nearly 3 times more coliforms than the standard m-Endo method (64). We also found that m-T7 agar recovered more verified coliforms from drinking water samples than the m-Endo, m-Endo + LTB pad, m-Endo + LA overlay, m-T7 + PLY pad or m-T7 anaerobic technique (64). In addition, five collaborating laboratories reported an average increase of 31% in coliform recoveries from a variety of contaminated surface and sewage samples using m-T7 agar compared to the standard m-Endo method. It should be emphasized that our results showed m-T7 agar performed best for isolation of coliforms from drinking water. Since m-T7 agar was specifically designed to recover injured coliforms from drinking water, further studies are needed to fully evaluate its potential. It is hoped that other investigators interested in coliform injury will use m-T7 and evaluate the results in relation to other coliform media.

The problem of enumerating injured fecal coliform bacteria has been recognized by a new section entitled "Stressed Organisms" (section no. 920) added to the 15th edition of Standard Methods for the Examination of Water and Wastewater (2). Included in this section are various resuscitation techniques for

Table 16. Formulation of m-T7 medium

Ingredient[a]	per liter distilled water
Proteose peptone #3	5 g
Yeast extract	3 g
Lactose	20 g
Tergitol 7 (25% solution)	.4 ml
Polyoxyethylene ether W1	5 g
Brom thymol blue	0.1 g
Brom cresol purple	0.1 g
Agar	15 g
Penicillin G[b]	1.0 mg
Final pH[c]	7.4

[a] All ingredients manufactured by Difco Laboratories except polyoxyethylene ether W1, Peniclin G, and brom cresol purple which were manufactured by Sigma Chemicals and tergitol 7 which was obtained from Baker Chemical Company.

[b] Penicillin G should be added to the medium after autoclaving (when tempered). Media prepared with Penicillin G should be used within one week when stored at 4°C.

[c] The medium was autoclaved at 121°C for 15 min and final pH was adjusted aseptically to 7.4 with 0.1 N NaOH.

fecal coliforms (44,68,91,99,110). We recently compared the effectiveness of m-T7 agar with some of the proposed resuscitation techniques for the recovery of injured fecal coliforms from chlorinated sewage effluents (65). Although a limited number of samples were examined, m-T7 agar preincubated at 37°C for 8 h before transfer to 44.5°C for an additional 12 h was superior for the recovery of verified fecal coliforms compared to any of the resuscitation techniques tested. This method (abbreviated m-T7 8 h, 37°C) produced counts 3.1 times greater than the standard m-FC technique and 1.7 times greater than the two layer enrichment, temperature acclimation procedure (65). Several advantages are immediately apparent for having one medium capable of performing both the total and fecal coliform tests. (i) The medium is simple. There are no overlay procedures, nor does the filter need to be transferred. (ii) The medium is convenient. Since contamination of drinking water supplies is usually transitory, it is neither expedient nor convenient to prepare another medium to assay for fecal pollution. In an emergency it would be easier to incubate the same medium at an elevated temperature for the fecal coliform tests. (iii) Finally, use of one medium for two tests could save money. Costs could be cut by not having to purchase a variety of media and chemicals and by saving time in media preparation. One disadvantage is that an incubator is required capable of adjusting temperatures after an 8 h interval. Although the technology is readily available and a similar device is on the market (Millipore Corp., Bedford, MA), we are not aware of such an incubator designed specifically for this method.

Importance of Enumerating Stressed Indicators

Fundamental questions arise when considering stressed indicator bacteria. Some of these include: How important are stressed coliform bacteria? Do they have any public health significance? If coliforms are injured, are pathogens injured as well? Are these pathogens still virulent? Should the maximum contaminant level be relaxed because of improved methods to detect injured coliforms? Although some investigators are quick to completely discount the importance of stressed indicator organisms (43), the fact remains that there are few data available to answer these questions. This informational void could be filled by state agencies and water testing labs performing parallel coliform analysis using m-Endo and m-T7 media. A limited survey of injured coliform in 254 samples from four distribution systems in Montana and Massachusetts was performed, including both chlorinated and unchlorinated drinking water supplies. The mean level of injury observed in those waters was 58% with results ranging from 43 to 86% (unpublished results) indicating that over half of the coliforms present were not recoverable with standard MF methods. In addition, epidemiological investigations should be initiated to further evaluate the significance of stressed indicator bacteria associated with waterborne outbreaks. Finally, increased awareness is needed among operators and managers of drinking water systems to the reality of injured coliforms and that methods are available to easily enumerate stressed indicator bacteria.

Despite the lack of data regarding the significance of stressed bacteria in drinking water supplies, there are several important reasons for enumeration of injured coliforms. The first of these is philosophical and relates to the sensitivity of the indicator concept (77). Fundamental to this concept is the view that an indicator system that is more encompassing is better than one that is more restrictive. Additionally, false negative results are to be avoided more than false positives, if one has to select the lesser of two evils. For example, in the distinction between sewage contamination and feces contamination, an effective indicator system will detect not only feces but also sewage contamination. The importance of this idea is that water contamination from sewage may vary widely in amounts of fecal material, and the variety of health threatening chemicals and infectious agents that may be transmitted through water is not exclusively associated with fecal material. A similar line of reasoning can be extended to the total coliform bacteria. Total coliforms are generally a good indicator of water supply treatment effectiveness, or of the intrusion of contaminated surface or groundwater into potable water supply. Enumerating all coliforms, including stressed or injured cells, provides a more encompassing and sensitive indicator system better capable of detecting the spectrum of potential health threats in contaminated water. This is particularly true since it is known that significant proportions of the coliform bacteria in surface waters and sewage that might intrude into potable water supplies are already injured (7,8,12,17,29,75,76,78). Enumeration of injured coliforms would therefore provide more accurate indication of contaminated drinking water.

The second reason for enumerating stressed indicator organisms comes from the observation that a number of Salmonella and poliomyelitis outbreaks occurred when coliforms were either undetected or detected only in low numbers by established techniques (11,70,82,105). During the last decade there has been a pronounced increase in both the number of cases and outbreaks of waterborne disease reported by the Centers for Disease Control (CDC) in Atlanta, GA. (111). Nineteen outbreaks were reported in 1971, involving 5,182 individuals while 32 outbreaks of waterborne disease with 11,435 cases were reported in 1978. More recent data also support this trend. During the period 1971-1978, 224 waterborne outbreaks of disease were reported in the U.S. Two deaths and 48,193 illnesses were associated with these outbreaks in which water was epidemiologically implicated as the vehicle of transmission. At the same time, some believe that as many as 90% of the outbreaks are unreported, since many people fail to associate their illness with contaminated drinking water. This increase might only be an apparent result of improved reporting or a real phenomenon resulting from the overloading of our treatment plants with source water of increasingly lower quality, inadequate or improper treatment, deterioration of the water treatment system, and complacency of the general public regarding water quality (111). In order to maintain a commitment to better quality drinking water, a more sensitive indicator system is needed to improve our surveillance system.

Finally, the last justification for enumerating injured coliforms is cost effectiveness. No additional costs are associated with the one step, m-T7 agar MF technique, since no additional manipulations are required. Only the purchase of a programmable incubator will be required for the implementation of the m-T7 8h 37°C fecal coliform procedure. In both cases, improved coliform recoveries are possible with little or no additional cost.

With improved enumeration procedures, treatment plants that produce quality drinking water now will see little change in their coliform recoveries (40,104). Water that is properly treated should not have any coliform occurrences (2,9,38,40) regardless whether they are stressed or not. Using more sensitive coliform methods to detect injured bacteria may, however, expose potable water facilities that are marginal in their treatment of drinking water. Generally, it is in these marginal treatment systems that waterborne outbreaks occur. Medical expenses and lost productivity alone has been estimated to cost millions of dollars each year. Implementation of more sensitive coliform methods could reduce the number of these outbreaks. This is probably the most effective rationale to support the need for enumeration of all viable coliform bacteria, including those that are injured.

Conclusions

The following conclusions can be drawn from data presented in this report:

1. Injury to indicator bacteria can result from a variety of sources including: chlorine and other disinfectants, heat, freezing, sunlight, pH, transition metals, antagonistic plate count organisms and possibly other undefined chemical and physical parameters. Injured bacteria exist in every aquatic environment examined so far.

2. A number of factors can influence the extent or severity of injury including: time and temperature of exposure, strain of organism, physiological state, concentration of nutrients, neutralization technique (i.e., EDTA to neutralize copper effects), diluent and choice of sample methodology.

3. Effective alternative methods exist that are specifically designed for the enumeration of injured total and fecal coliforms.

4. While little information is available to answer important questions concerning injured indicator bacteria, several reasons were presented why stressed coliforms should be enume-

rated.

Recommendations

1. Water operators and managers should become aware of the reality of injured coliforms. Injured indicator bacteria exist in every aquatic environment studied thus far, and these organisms may represent about half of the coliforms present.

2. Laboratories examining potable water should perform parallel coliform analyses using m-Endo and m-T7 agars in order to assess the extent of occurrence of injured coliforms in their systems.

3. Epidemiological investigations should be initiated to further evaluate the significance of stressed indicator bacteria associated with waterborne outbreaks.

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APPENDIX

EVALUATION OF m-T7 AGAR BY COLLABORATING LABORATORIES

Several collaborators have reported the results of their evaluation of m-T7 agar as a total coliform medium. Guidelines of the national equivalency program were sent to these investigators so the medium could be evaluated on a national basis. Individuals from 37 different laboratories indicated they would cooperate by evaluating m-T7.

1. Ed Means from the Metropolitan Water District of Southern California, compared the recovery of coliforms using m-T7 agar, m-Endo broth and m-Endo agar (Table 1). The efficiency of the different media was tested on treatment plant influent samples consisting of a 50:50 blend of State Project water and Colorado River water. Coliforms were verified in LTB. m-T7 agar was more efficient in recovering coliforms than either of the other two methods; isolating an average of 58.6% more coliforms than m-Endo broth and 34.5% more coliforms than m-Endo agar from four surface water samples. A few coliforms have been identified from these samples and all have been "typical coliforms" (*Escherichia coli* and *Citrobacter sp.*). All typical yellow colonies were readily apparent and invariably confirmed in lauryl tryptose broth, while distinct brown-yellow colonies were often isolated and frequently confirmed as coliforms.

2. John Wierenga and Bob Kneibel from City of Grand Rapids, MI., reported a 40% increase in coliform recoveries using m-T7 agar (Table 2). However, they reported problems with difficulty in determining yellow and non-yellow colonies, some overgrowth problems and low verification rates for both m-T7 agar and m-Endo agar. Problems were worst when testing water samples from Lake Michigan. No drinking water samples were evaluated.

3. Ellen Vigna and Barbara Green from Millipore Corp., Bedford, Mass., compared m-T7 and m-Endo agar for recovery of coliforms from raw sewage and surface water samples (Table 3). From the four samples, m-T7 agar recovered an average of 48.8 coliforms while m-Endo recovered 30.9. This difference represents a 42% increase in coliform recovery. Difficulties experienced included a variety of colors ranging from pale yellow to yellow-green and crowded colonies.

4. A presentation at the annual meeting of the American Society for Microbiology (New Orleans, Louisiana; 1983) was given by Robert Duncanson and Victor Cabelli (Q 22) where these investigators compared mCm, m-Endo and m-T7 agar. m-T7 agar recovered

Table A-1. Comparison of Coliform Recoveries from Metropolitan Water District of Southern California Source of Water.

Date	Verified Coliform Counts[a]			
	No. of replicates (28)[b]	m-Endo broth	m-Endo agar	m-T7 agar
3-16-82	10	1.4	2.6	5.3
3-18-82	5	1.0	2.8	3.6
3-22-82	5	1.0	0.4	0.6
3-23-82	8	1.3	2.0	2.1
Mean	7	1.2	1.9	2.9

[a] Because different dilutions were used on some samples, counts are represented as relative values, irrespective of dilution.

[b] Total number of replicate counts was 28.

Table A-2. Comparison of Coliform Recoveries from Lake Michigan and Grand River, MI.

		Verified Coliform Counts (per 100 ml)		
Date	No. of replicates	M-Endo agar	m-T7 agar	MPN
10/26/82[a]	4	2[c]	5	5
10/27/82[a]	4	3[c]	4	8
11/1/82[a]	4	6[c]	6	7
10/26/82[b]	4	5	5	ND[d]
10/27/82[b]	4	0	5	ND[d]

[a] Samples from Lake Michigan

[b] Samples from the Grand River

[c] Unverified counts

[d] ND; not done

Table A-3. Comparison of Coliform Recoveries from Sewage and Surface Water Samples, Bedford, Mass.

		Coliform Counts[a]	
Date	Location	m-Endo agar	m-T7 agar
8/13/82	Millipore Brook	21.0 (2.1)[b]	22.7 (11.9)
8/13/82	Raw Sewage, Concord	31.0 (1.7)	28.6 (13.8)
8/18/82	Raw Sewage, Concord	14.0 (1.0)	33.3 (3.0
8/25/82	Raw Sewage, Concord	57.6 (5.0)	90.6 (2.1)

[a] Because different dilutions were used on some samples, counts are represented as relative values, irrespective of dilution. It is unknown whether these counts are verified coliform counts.

[b] Numbers in parentheses are standard deviations based on three replicate analyses.

63% more coliforms than m-Endo medium and slightly more coliforms than mCm agar from 12 samples of sewage, surface, and contaminated well water. Background colonies on m-T7 agar averaged 70 colonies per filter (note: Standard Methods; section 909A, specifies "no more than 200 colonies of all types" per filter). In this study by Duncanson and Cabelli, the ratio of background organisms per coliform was 4.4 for m-T7 agar but 5.7 for m-Endo agar. On a per coliform basis, m-T7 agar had less background colonies than m-Endo agar. In our study we found similar results. The ratio of background bacteria per coliform for three surface water samples was 5.4 for m-T7 agar and 7.2 for m-Endo agar. Data is not available for the ratio in drinking water.

5. Robert Bordner and John Haines; EPA Cincinnati, reported 26% lower coliform counts using m-T7 agar than m-Endo agar from surface and sewage treatment plant effluents (Table 4). Verification rates ranged from 0 to 75% from m-T7 agar and 0 to 49% for m-Endo agar. Major problems consisted of inability to distinguish yellow from non-yellow colonies, background growth and confluency. No drinking water samples were examined.

Overall, a 31% increase was observed when m-T7 agar was used to recover coliforms from sewage and surface waters. Excluding the one report of lower recoveries, a 45% increase was observed. Many investigators reported a range of colony colors on m-T7 agar ranging from yellow to yellow-green or brownish yellow. This commonly occurs because a coliform and a non-coliform colony have grown together. With experience this is easy to spot, but caution should be used when verifying with the coliform tested directly off the filter because the mixed culture could give a false negative reaction.

Other important considerations are: (i) proper media composition and pH; (ii) counting and picking colonies for verification using 15x magnification; and (iii) activity of penicillin G solution. One incident in our lab showed that penicillin G stored frozen for 9 months and left at room temperature for several hours had markedly reduced activity. Initial difficulties distinguishing coliform from non-coliform colonies are not unexpected with people not experienced with a new medium.

Our results showed that m-T7 agar performed best for isolation of coliforms from drinking water. Presently, we are not aware of any comparison of m-T7 and m-Endo agar in drinking waters. m-T7 agar was designed to recover injured coliforms from drinking water and further research should be done in order to fully evaluate its potential.

Table A-4. Comparison of Coliform Recoveries from Sewage Treatment Plant Effluents and Ohio River Water.

Source[c]	m-Endo Agar	Percent Verification	m-T7	Percent Verification
Ludlow Springs	4.03×10^3	ND[a]	1.97×10^3	ND
Public Landing, OR	2.63×10^4	15%	1.73×10^4	11%
Mill Creek STP	9.30×10^4	47%	4.40×10^4	37%
Mill Creek STP	4.30×10^5	ND	Confluent	ND
Muddy Creek STP	9.07×10^4	49%	4.13×10^4	75%
			[b]6.06×10^4	52%
Mill Creek STP	10	0%	10	0%
Muddy Creek STP	80	5%	57	10%
			[b]76	15%

[a] ND; not done

[b] Prepared medium held in refrigerator two weeks.

[c] The temperatures of all source waters were from 19 to 25°C except Ludlow Springs at 12.5°C. Public Landing is the boat landing on the Ohio River in downtown Cincinnati. Muddy Creek STP receives about 90% domestic sewage. Mill Creek STP receives about 90% industrial waste.